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**“Protein kinase inhibitors for the treatment
of thyroid cancer: molecular mechanisms of
resistance”**

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications:

- I. Muthu et al. Molecular mechanisms of thyroid cancer cell resistance to BRAF chemical blockade. Manuscript in preparation (main body of the Dissertation)
- II. De Falco et al. Ponatinib is a novel potent RET kinase inhibitor. Manuscript in preparation (main body of the Dissertation)
- III. Castellone MD, De Falco V, Rao DM, Bellelli R, Muthu M, Basolo F, Fusco A, Gutkind JS, Santoro M. The beta-catenin axis integrates multiple signals downstream from RET/papillary thyroid carcinoma leading to cell proliferation. *Cancer Res.* 2009 Mar 1;69(5):1867-76 (attached at the end of Dissertation)
- IV. Castellone MD, Verrienti A, Magendra Rao D, Sponziello M, Fabbro D, Muthu M, Durante C, Maranghi M, Damante G, Pizzolitto S, Costante G, Russo D, Santoro M, Filetti S. A novel de novo germ-line V292M mutation in the extracellular region of RET in a patient with pheochromocytoma and medullary thyroid carcinoma: functional characterization. *Clin Endocrinol (Oxf).* 2010 Oct;73(4):529-34 (attached at the end of Dissertation)

LIST OF ABBREVIATIONS

ATC	Anaplastic Thyroid carcinoma
BRAF	B-type RAF family kinase
CCH	C-cell hyperplasia
EGFR	Epidermal growth factor receptor
FGFR	Fibroblast growth factor receptor
FTA	Follicular Thyroid Adenoma
FTC	Follicular Thyroid carcinoma
FV-PTC	Follicular-variant PTC
GDNF	Glial cell line-derived Neurotrophic Factor
GFR α	GDNF-family receptor α
MAPK	Mitogen-Activated Protein Kinases
MEN2	Multiple Endocrine Neoplasias type 2
MTC	Medullary Thyroid carcinoma
NIS	Sodium-iodine symporter
OS	Overall Survival
PDGFR	Platelet-derived Growth Factor Receptor
PDTC	Poorly differentiated Thyroid Carcinoma
PFS	Progression-free survival
PIK3CA	Phosphoinositide-3 kinase
PKI	Protein kinase inhibitors
PTC	Papillary thyroid Carcinoma
RET	REarranged during Transfection
RET/PTC	RET/Papillary Thyroid Carcinoma
RTK	Receptor tyrosine Kinase
TCV-PTC	Tall-cell variant PTC
VEGFR	Vascular endothelial growth factor receptor
WDTC	Well differentiated thyroid carcinoma
WT	Wild type

ABSTRACT

Though most types of thyroid cancer are cured by surgery and adjuvant radio-iodine therapy and TSH suppression, patients with radio-iodine refractory thyroid carcinoma as well as with medullary thyroid carcinoma, a neuroendocrine malignancy that does not express iodine transporter, are difficult to treat because of the lack of effective systemic treatment. Therefore, there is an urgent need of novel therapeutic measures for these patients. Thyroid cancer is frequently associated to the oncogenic conversion of protein kinases such as RET (medullary thyroid cancer) and BRAF (papillary and undifferentiated thyroid cancer). Therefore, protein kinase small molecule inhibitors (PKI) have been considered as promising novel agents for the treatment of thyroid carcinoma. Compounds that revealed good RET and BRAF activity both in clinical (vandetanib for RET) and in pre-clinical (vemurafenib for BRAF) settings have been identified. Recently, vandetanib was approved for patients with medullary thyroid carcinoma and vemurafenib was approved for patients affected by melanoma. Cancer patients may be refractory to PKI or develop secondary resistance after an initial response. Two general mechanisms can explain resistance: i) the presence of mutations in the kinase that blocks binding to the PKI; ii) the oncogenic switch to another pathway that rescues cancer cells from kinase blockade. In this dissertation, we have addressed mechanisms of resistance in the case of RET PKI (for the treatment of medullary thyroid carcinoma) and BRAF PKI (for the treatment of radio-iodine refractory thyroid carcinoma). Here we show that: i) ponatinib is a novel RET PKI and is able to overcome RET resistance mediated by mutations in RET V804 and Y806; ii) thyroid cancer cells escape treatment with vemurafenib by switching to another signaling pathway and that inhibitors of the IGF1R/PI3K/AKT signaling cascade may be exploited to overcome resistance of thyroid cancer cells lines to BRAF PKI.

1. INTRODUCTION

1.1 Thyroid gland

The thyroid gland is composed of two distinct hormone-producing cells: follicular cells (producing thyroid hormones) and parafollicular C cells (producing calcitonin). Most of the thyroid follicular cells are derived from the endodermal diverticulum, whereas a few of them derive from the ultimobranchial body. The parafollicular cells are neural crest derived; they originate from the ultimobranchial body and are interspersed in small groups among the follicles in the intermediate part of the thyroid lobes (DeLellis et al. 2004; Kondo et al. 2006). Follicular cells concentrate inorganic iodide from the bloodstream to produce the hormones thyroxine (T₄) and its bioactive derivative triiodothyronine (T₃) from thyroglobulin. The parafollicular C-cells secrete calcitonin, which is involved in bone metabolism. Calcitonin secretion is stimulated by elevated calcium concentration in the serum.

1.2 Thyroid Carcinoma

Benign thyroid lesions are typically solitary adenomas. They can be clinically silent or hyper-functioning (referred to as a toxic thyroid adenoma), thereby causing hyperthyroidism. Gain-of-function mutations of TSHR (~80%) and GNAS1, encoding G α_s , (~25%) are the most common genetic lesion in functional thyroid adenomas, whereas these genetic lesions did not occur in thyroid malignancies (Kondo et al. 2006).

Malignant thyroid lesions are the most common cancers of endocrine organs and represent approximately 1% of newly diagnosed cancer cases (DeLellis et al. 2004), with an incidence rate stably increasing over the past few decades (Jemal et al. 2010). Tumors deriving from follicular cells represent more than 95% of thyroid cancers, whereas only 3-5% of them arise from C-cells. On the basis of histological and clinical parameters, malignant follicular-derived lesions are classified into well-differentiated carcinomas (WDTC) in turn including papillary (PTC) and follicular (FTC) carcinomas, poorly differentiated carcinomas (PDTC), and anaplastic carcinomas (ATC) (DeLellis et al. 2004, Kondo et al. 2006).

1.2.1 Papillary Thyroid Carcinoma (PTC)

PTC is the most prevalent thyroid cancer subtype (80% of cases) (DeLellis et al. 2004). PTC is characterized by typical features of the cell nuclei, including enlarged, vesicular and overlapping nuclei with clearing, fine dusty chromatin, grooves, inclusions and single or multiple micro- or macronucleoli. PTC is closely linked to radiation exposure and its incidence sharply increased in children after the Chernobyl accident (Williams 2008).

Over 70% of PTC feature genetic alterations causing activation of the MAPK pathway (Kondo et al. 2006; Nikiforov et al. 2011) (**Table 1**). PTC is characterized by mutations of various MAPK components, including rearrangements of the RET (REarranged during Transfection) tyrosine kinase receptor, or more rarely NTRK1, or activation of BRAF (B-type RAF family kinase). RET/PTC and BRAF alterations are mutually exclusive in PTC, suggesting that mutations at more than one of these sites are unlikely to confer additional biological advantage, a fact that is consistent with the overlap of the signaling pathways of these oncoproteins. Specific PTC subtypes have different mutation patterns. For example, FV-PTC (follicular-variant PTC), when encapsulated, frequently (up to 40%) harbors mutations in RAS (Rivera et al. 2010). In contrast, infiltrative FV-PTC often (25%) harbors BRAF mutations. BRAF mutant FV-PTC may harbor the K601E or other alternative mutations rather than the classical BRAF V600E (Xing 2007). Compared to FV-PTC and classical PTC, TCV-PTC (tall-cell variant PTC) shows a high (up to 80%) proportion of BRAF V600E positive cases (Nikiforova et al. 2003).

1.2.2 Follicular Thyroid Carcinoma (FTC)

FTC accounts for about 10% of all thyroid malignancies, though the incidence may be declining relative to PTC (DeLellis et al. 2004). FTC can be hardly differentiated from FTA (Follicular Thyroid Adenoma) and possibly some FTC may have evolved from pre-existing FTA. FTC shares some genetic alterations with FTA (Kondo et al. 2006). One group of FTC is associated with RAS gene mutations while the RAS mutation-negative FTC subgroup often features gene rearrangements of PPARG, most commonly the PAX8-PPARG fusion (**Table 1**). RAS and PAX-PPARG do not overlap and together they explain up to 80% FTC cases (Nikiforov et al. 2011).

1.2.3 Poorly-Differentiated Thyroid Carcinoma (PDTC)

PDTC are rare neoplasms characterized by a partial loss of differentiation and less favorable prognosis in comparison with WDTC (Volante et al. 2010). Diagnostic criteria for PDTC include the presence of a solid-trabecular-insular pattern of growth, absence of the nuclear features typical of PTC and the presence of at least one of these features: convoluted nuclei (small hyperchromatic nuclei with convolutions of the nuclear membrane); high mitotic; and tumor necrosis (Volante et al. 2010). PDTC have genetic features intermediate between WDTC and ATC. Similar to WDTC, PDTC harbours mutations in RAS or, less frequently (15%) in BRAF (Table 1).

Table 1: Genetic lesions associated to thyroid carcinoma

Genetic alteration	Function	PTC	FTC	PDTC	ATC	MTC
RET/PTC rearrangement	Receptor tyrosine kinase	20%	—	rare	—	—
TRK-T rearrangement	Receptor tyrosine kinase	5-13%	—	—	—	—
RET mutation	Receptor tyrosine kinase	—	—	—	—	50% MEN2: 95%
BRAF mutation	Serine threonine kinase	45%	—	15%	44%	—
RAS mutation	Small GTPase	10%	50%	44%	20-60%	—
PIK3CA mutation	Lipid kinase	rare	rare	rare	20%	—
PPARG rearrangement	Nuclear receptor	—	35%	rare	—	—
TP53	Transcription factor	rare	rare	15-30%	60-80%	rare

Similar to ATC, PDTC features in some cases mutations in TP53 (15-30%) or in PIK3CA (phosphoinositide-3 kinase) and its downstream effector AKT (Ricarte-Filho et al. 2009). RAS (predominantly NRAS codon 61) mutations are the most prevalent genetic lesions found in PDTC and are being identified in up to 44% of cases (Ricarte-Filho et al. 2009; Volante et al. 2010).

1.2.4 Anaplastic Thyroid Carcinoma (ATC)

ATC accounts for 2–5% of all thyroid cancers. More than 25% of ATC patients have coincidentally detected well-differentiated (PTC or FTC) carcinoma, suggesting their tumors derive from pre-existing PTC or FTC (DeLellis et al. 2004; Kondo et al. 2006). In up to 75% of ATC cases, there is an evidence of distant metastases at diagnosis and the median survival of ATC patients is 5 months (Smallridge and Copland 2010). Chemotherapy is ineffective in ATC and only partial responses are obtained in less than 25% of cases (Smallridge and Copland 2010).

Genetically ATC is associated with BRAF and RAS mutations whereas RET/PTC and TRK-T mutations are not found (**Table 1**). BRAF mutations are mainly found in samples containing a PTC component, thus supporting the notion that these ATC may derive from a pre-existing PTC (Nikiforova et al. 2003). Additional mutations identified in ATC include mutations in beta-catenin and in TP53 tumor suppressor, lesions that are rarely found in WDTC (**Table 1**). TP53 loss-of-function is likely critical for the establishment of the ATC phenotype. In a recent gene expression profiling paper, a set of chromosomal instability and cell proliferation genes were found up-regulated in ATC and such upregulation was mechanistically linked to TP53 mutation (Salvatore et al. 2007). Finally, mutations targeting components of the phosphoinositide-3 (OH) kinase (PI3K) signaling cascade were found in ATC and less frequently in WDTC (Saji and Ringel 2010).

1.2.5 Medullary Thyroid Carcinoma (MTC)

MTC accounts for about 5% of all thyroid cancers (Schlumberger et al. 2008). MTC is sporadic in about 75% of cases, and in the others it is a component of the autosomal dominant cancer syndrome known as “multiple endocrine neoplasia type 2” (MEN2). MEN2 includes three subtypes: MEN2A (about 80% of cases), MEN2B and familial MTC (FMTC) (Waguespack et al. 2011). MEN2-associated hereditary MTC is typically bilateral and multicentric and it is usually preceded by multifocal C-cell hyperplasia (CCH). In MEN2A, MTC is associated with pheochromocytoma, parathyroid hyperplasia and, rarely, cutaneous lichen amyloidosis (CLA) and Hirschsprung’s disease. In MEN2B, MTC is associated with pheochromocytoma, multiple ganglioneuromas of the lips and gastrointestinal tract and marfanoid body habitus. Our knowledge of the genetic lesions associated with MTC is still very limited. Genes commonly mutated in other types of cancer - as well as other thyroid cancers - like BRAF, PIK3CA and TP53 - are not commonly mutated in MTC. Mutations in the RET proto-oncogene is present in about half of sporadic cases and virtually all familial cases (**Table 1**). Importantly, RET mutations correlate with a more aggressive

MTC phenotype and blood screening for RET mutations can be used to identify patients with familial form of MTC (Elisei et al. 2008; Romei et al. 2011). In some cases, RET gene amplification has been reported (Ciampi et al. 2012). Recently, RAS mutations have been found in RET-negative MTC cases (Moura et al. 2011)

1.3 RET

The RET proto-oncogene is located on chromosome 10q11.2 and encodes a transmembrane receptor tyrosine kinase (RTK) with four cadherin-related motifs in the extracellular domain (Santoro et al. 2006). RET is normally expressed in the developing central and peripheral nervous system and is an essential component of a signaling pathway required for renal organogenesis and enteric neurogenesis. RET is normally expressed at high levels in C-cells, but not in follicular cells (Santoro et al. 2006). Glial cell line-derived neurotrophic factor (GDNF)-family ligands and GDNF-family receptor α (GFR α) bind the extracellular domain of RET inducing its dimerization. This active form of RET autophosphorylates specific tyrosine residues within its intracellular domain, that function as binding sites for signaling molecules containing phosphotyrosine-binding motifs (SH2 or PTB), thereby activating several signaling pathways (Santoro et al. 2006). Through phosphorylated Y1062, RET binds SHC and FRS2, that recruit Grb2-SOS complexes leading to the activation of the RAS-RAF-ERK cascade (Borrello et al. 1994). Through pY1062, RET is also able to activate the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Santoro et al. 2006) (**Figure 1**).

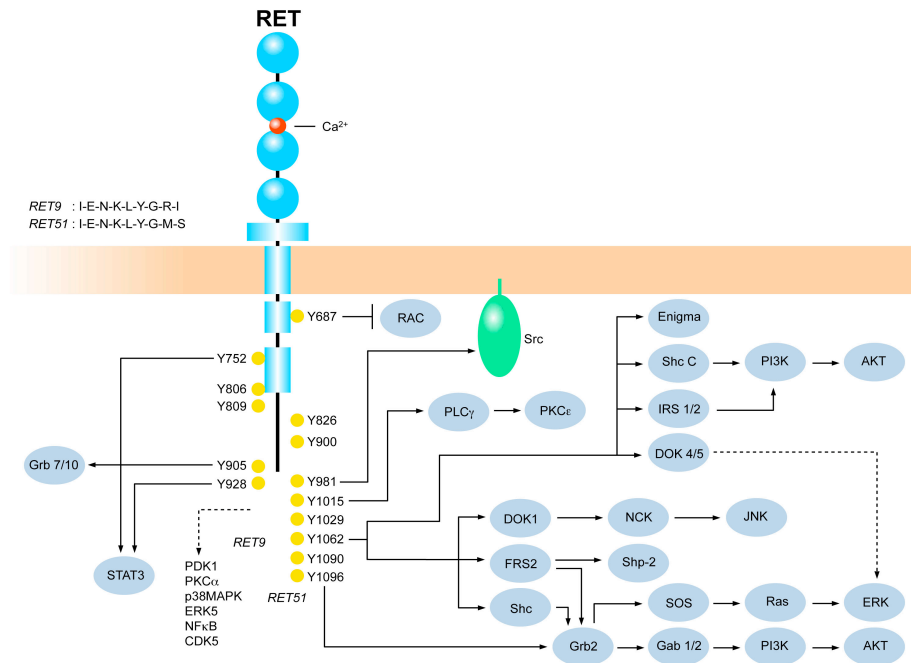


Fig. 1: The RET structure and signaling network. The structure of the RET protein and its signaling network with the docking sites and targets.

As mentioned above, gain-of-function mutations of RET are involved in sporadic MTC and FMTC, including MEN2A and MEN2B (**Figure 2**). In 95% of cases, MEN2B patients carry the M918T mutation in the RET kinase domain; the remaining fraction harbors the A883F substitution or other rare mutations. In 98% of MEN2A and 90% of FMTC cases, mutations affect one of the five cysteines in the extracellular cysteine-rich domain of RET. The remaining cases have mutations in few other codons mainly located in RET exons 13, 14, or 15 (**Figure 2**). Somatic mutations of RET, which mainly target V804, M918 and E768, are found in about half of sporadic MTC. All these mutations activate RET converting it into a dominantly transforming oncogene (Santoro et al. 2006; Wells et al. 2009). However, the various mutations have different oncogenic potential and this represents the basis for the different timing of prophylactic thyroidectomy according to the MTC guidelines (Pacini et al. 2010; Kloos et al. 2009; Waguespack et al. 2011).

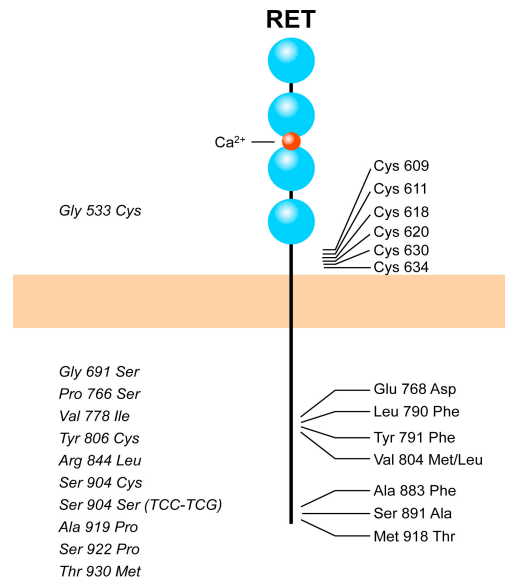


Fig. 2: Schematic representations of most common RET mutations associated to MTC.

Conversely, PTCs are characterized by chimeric oncoproteins named RET/PTC, originating from the in-frame fusion of the RET tyrosine kinase encoding domain and 5'-ter of different partner genes (Kondo et al. 2006) (**Figure 3**). RET/PTC lacks RET signal peptide and transmembrane domains but retains the kinase domain and most of the autophosphorylation sites, thereby allowing downstream signaling. By providing an active transcriptional promoter, RET/PTC rearrangements enable thyroid expression of the chimeric RET/PTC oncoproteins. To date, more than 10 RET/PTC rearrangements have been described; the most frequent are RET/PTC1, which involves RET and H4 (CCDC6) genes and RET/PTC3, between RET and RFG (RET-Fused Gene) (also named NCOA4, ELE1 or ARA70) (Kondo et al. 2006). RET/PTC rearrangements are found in about 20% of PTC, with higher prevalence in classic form than in follicular variant (Kondo et al. 2006; Nikiforov et al. 2011). Their prevalence is significantly higher in young age patients and in patients with a history of accidental or therapeutic radiation exposure.

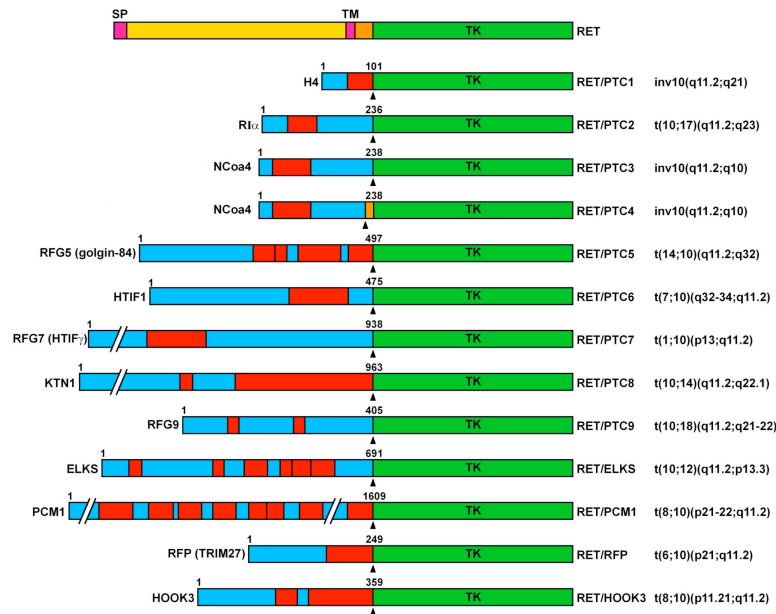


Fig. 3: Schematic representation of RET/PTC rearrangements in PTC

Moreover, at a variance from BRAF (see below), RET/PTC doesn't seem to be a negative prognostic factor for PTC (Xing 2007). The low prevalence of expression of RET/PTC oncoproteins in PDC and ATC suggests that they do not play a prominent role in thyroid tumor progression (Nikiforov et al. 2011).

1.4 BRAF

BRAF (7q24) is a member of the RAF kinase family of serine/threonine-specific protein kinases, comprising three members: ARAF, BRAF and CRAF (or RAF-1) (Chong et al. 2003). Homologues of the three corresponding genes are found in all vertebrates, while a single RAF gene exists in invertebrates (for example, DRAF in *Drosophila melanogaster* and lin-45 in *Caenorhabditis elegans*), whose sequence is most closely related to BRAF. The RAF gene products are RAS effectors participating to the ERK (MAPK) signaling pathway. ERK/MAPK pathway, in turn, connects extracellular signals to transcriptional regulation (Schubbert et al. 2007). RAS is activated by growth factors and hormones and in turn activates multiple downstream pathways controlling cellular survival, proliferation and differentiation. In its active GTP-bound state it binds to RAF proteins (also named MAPKKK), thereby recruiting them to the plasma membrane. Once localized at the membrane, RAF kinases are activated by a series of phosphorylation and

dephosphorylation events. BRAF and CRAF can also heterodimerize in response to mitogenic signals and the activity of the complex requires at least one of the two monomers to be active. Activated RAF proteins phosphorylate and activate MEK1 and MEK 2 (MAPKK), which in turn phosphorylate and activate ERK1 and ERK2 (MAPK). ERKs phosphorylate several cytoplasmic and nuclear targets, including transcription factors such as Ets-1, c-Jun and c-Myc. The multiple steps in the RAS/RAF/MEK/ERK pathway provide a mechanism of signal amplification as well as a platform for signal modulation by other factors (**Figure 4**).

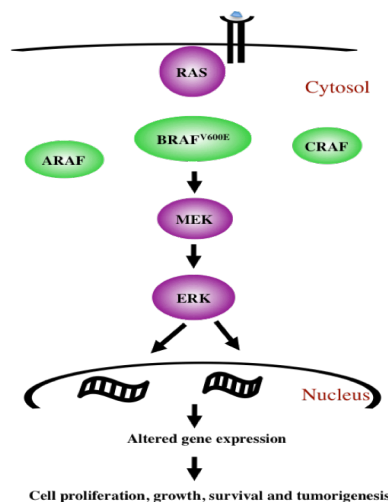


Fig. 4: Schematic representation of MAPK pathway.

The RAF proteins display a common architecture with two N-terminal regulatory domains (CR1 and CR2) and one kinase C-terminal domain (CR3). The three RAF members show differences in tissue distribution and activation modalities. Importantly, several residues must be phosphorylated in ARAF and CRAF for full activation, while some of them are constitutively phosphorylated or replaced with phosphomimetic residues in BRAF. This makes BRAF activation more rapid and efficient than that of CRAF or ARAF (**Figure 5**). Accordingly, BRAF has a strong basal kinase activity and is the most potent activator of MEK in many cell types; this may account for the high prevalence of mutations in BRAF rather than other RAF family members, in human tumors (Dhomen and Marais 2007). In fact, BRAF can be activated by single amino acid substitutions, while CRAF and ARAF require at least two mutations for oncogenic conversion (Dhomen and Marais 2007).

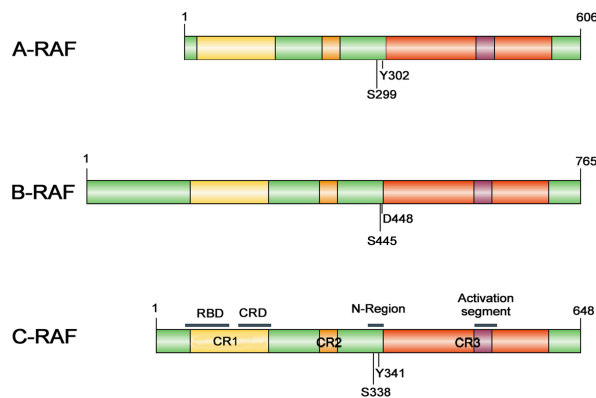


Fig. 5: Schematic representation of the RAF proteins. The three conserved regions: CR1 (yellow), CR2 (orange) and CR3 (red) are highlighted. The amino acids indicated below the individual isoforms refer to known regulatory phosphorylation sites. CR1 contains the RAS-binding domain and CR3 contains the catalytic domain (the activation segment is highlighted in pink).

1.4.1 BRAF V600E mutation

The most common oncogenic mutation in BRAF is the replacement of valine 600 with a glutamic acid (**Figure 6**). BRAF V600E is an oncogenic protein with markedly elevated kinase activity that over-activates the MAP kinase pathway (Davies et al. 2002; Wan et al. 2004). The BRAF V600E mutation occurs in approximately 8% of human cancers and ranks after RAS mutation (15%) as the second most common somatic mutation observed in human cancer. In comparison to wild-type BRAF, BRAF V600E transforms normal cells and does not require RAS function to induce growth of human cancer cells.

The aliphatic side chain of Val600 interacts with the phenyl ring of Phe466 in the P loop of the BRAF kinase. In the V600 mutants, the medium sized hydrophobic Val side chain is replaced with a larger and charged residue (most commonly Glu, but some times also Asp, Lys, or Arg); this is expected to destabilize the interactions that maintain the DFG motif in an inactive conformation, thereby flipping the activation segment into the active position, thereby activating the kinase in a RAS-independent manner and conferring transforming activity.

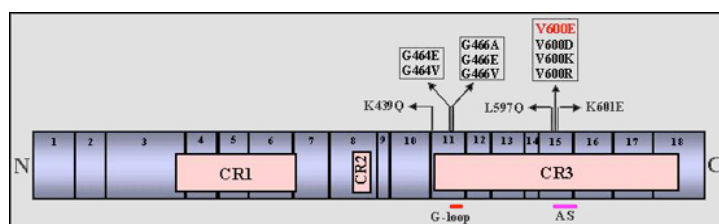


Fig. 6: BRAF mutations found in cancer. The black arrows indicate the mutations. The mutations are boxed when they target the same amino acid. The hotspot mutation V600E is in red. BRAF exons are also indicated, as well as conserved regions CR1, CR2 and CR3. A conserved glycine motif (G-loop) in exon 11 is indicated with a red bar and the activation segment (AS) in exon 15 with a pink bar: the V600E mutation targets the AS. C: Carboxyl-terminal; N: Amino-terminal.

BRAF V600E kinase function is activated by approximately 500-fold with respect to the wild type protein. Moreover, it induces constitutive ERK signaling through hyperactivation of the MEK–ERK pathway, thereby stimulating proliferation, survival and transformation (Davies et al. 2002). V600E mutation has been observed in melanoma (80%), papillary thyroid cancer (44%) and in other tumors (0-18%), including colorectal (5%) and lung cancer (1-3%) (Namba et al. 2003).

Other BRAF mutations include R461I, I462S, G463E, G463V, G465A, G465E, G465V, G468A, G468E, N580S, E585K, D593V, F594L, G595R, L596V, T598I, A727V, and a few others. Most of these mutations are clustered in two regions, the glycine-rich P-loop of the N-lobe and the activation segment and flanking regions (Wan et al. 2004) (**Figure 6**). Surprisingly, the activity of some cancer-associated BRAF mutants is impaired rather than potentiated. However, these impaired BRAF mutants can still activate MEK through CRAF. This BRAF/CRAF interaction, is followed by allosteric activation and transphosphorylation of CRAF, thus resulting in increased signaling to MEK and ERK (Dhomen and Marais 2007) (**Figure 7**). This mechanism might have important implications in a therapeutic setting; indeed, it may explain why cancers with wt BRAF, particularly if positive for RAS mutations, are insensitive to BRAF chemical inhibitors (Flaherty et al. 2010). It may be expected that through the BRAF-CRAF dimerization, BRAF inhibitors may even result in an activation rather than block of the MEK pathway.

As mentioned above, BRAF V600E mutation is predominantly seen in melanoma (Davies et al. 2002) and papillary thyroid carcinoma (Cohen et al. 2003; Kimura et al. 2003; Namba et al. 2003; Soares et al. 2003; Xing et al. 2004). However, in June 2011, a team of Italian scientists performed massive parallel sequencing to pinpoint V600E mutation in 100% of cases of Hairy cell leukaemia (Tiacchi et al. 2011).

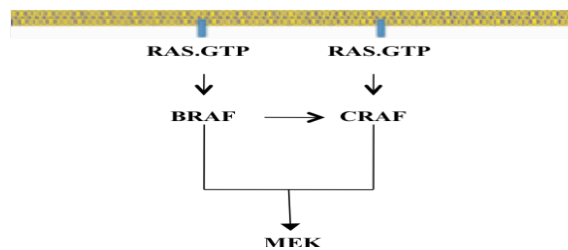


Fig. 7: RAS mediates BRAF-CRAF crosstalk. CRAF activity can be stimulated, in the presence of active RAS, through dimerization with BRAF. This mechanism functions also when BRAF is targeted by loss-of-function mutations. This might explain why in some rare cases BRAF mutations found in cancer are inactivating rather than activating mutations.

1.5 BRAF V600E mutation in melanoma

Melanoma is one of the most highly metastatic cancers. It arises from melanocytes, and in most of the cases it affects the basal layer of the epidermis. Melanoma represents 2% to 3% of malignant tumors in both United States and Northern Europe. The incidence of melanoma has doubled in the last 20 years; a 4% increase every year is documented in the United States (Jemal et al. 2010). Cutaneous melanoma is the most frequently occurring melanoma; however, melanoma can occur in other sites, such as the uvea, the most frequent melanoma location after skin (Grin et al. 1998). Melanoma has metastatic potential greater than other skin cancers such as basal cell carcinoma and squamous cell carcinoma. Approximately, 80% of melanomas are detected at an early stage in which the tumor is localized in the primary site and can be excised (Russo et al. 2009). Melanomas can be classified into 2 groups, those in the radial growth phase (RGP) and those in the vertical growth phase (VGP). RGP melanomas are early lesions and confined, for the most part, to the epidermis with a low probability of dissemination. Once progressed to the VGP state, tumor cells are highly tumorigenic, and characterized by dermal invasion, and distant metastases (Balch et al. 2001; Veronesi and Cascinelli 1991).

Metastatic melanoma is refractory to conventional therapies and has a very poor prognosis, with a median survival rate of only 6 months (Miller and Mihm 2006). Chemotherapy is not very effective for melanoma. In a prospective randomized controlled trial, adjuvant high-dose interferon was shown to increase progression-free (PFS) and overall (OS) survival (Kirkwood et al. 1996). Metastatic melanoma is rarely curable with standard chemotherapy, although high-dose interleukin-2 (IL-2) has been reported to produce durable responses in a small number of patients (Atkins et al. 2000).

In this framework, the most promising melanoma treatment might be that targeted to those mutations in transduction/survival pathways that are common in melanomas. These targeted therapies may include selective inhibitors of the RAF and MEK kinases and inhibitors of the PI3K pathway (Russo et al. 2009). Based on the high prevalence of BRAF mutations in melanoma, RAF inhibitors have been considered for targeted therapy for melanoma. Consistently, a direct correlation has been found between the presence of mutated BRAF and melanoma stage, with a higher incidence of BRAF mutations in VGP compared to RGP tumors (Dong et al. 2003).

1.6 BRAF V600E mutation in thyroid cancer

Papillary thyroid carcinoma (PTC) comprises almost 80% of all thyroid cancers. As mentioned above, in PTC, mutations of genes encoding effectors of the mitogen-activated protein kinase (MAPK) pathway are present in about 70% of all cases, and include mutually exclusive mutations in BRAF, RAS, and RET/PTC or NTRK (Namba et al. 2003; Xing et al. 2004; Kimura et al. 2003). As in melanoma, most common BRAF mutation in PTC is V600E. An intrachromosomal rearrangement between BRAF and AKAP9, leading to the expression of a constitutively active form of BRAF has been described, though very rarely, in radiation-induced PTC (Ciampi et al. 2005). Prior exposure to ionizing radiation during childhood predisposes to development of PTC and to RET rearrangements and, to a lesser extent, NTRK or BRAF intrachromosomal inversions. These rearrangements are also found in pediatric PTC without a history of radiation exposure. In contrast, point mutations in BRAF are rare in this population (4–6%) (Xing 2005). Thus, somatic mutation of BRAF is the most common early genetic event causally associated with the development of PTC in adult patients without a history of radiation exposure.

BRAF mutation (in most of the cases: V600E) occurs in PTC and PTC-derived ATC, with a prevalence of 44% in the former and 24% in the latter (Xing 2005). In contrast, BRAF mutation does not occur in FTC or other types of thyroid tumors. PTCs with BRAF mutation have distinct phenotypic and biologic properties. The tall-cell variant (TCV-PTC), an aggressive variant of PTC, usually harbors BRAF mutation (Adeniran et al. 2006). PTCs with BRAF mutation commonly present at an advanced stage, usually with extrathyroidal extension. Some studies suggest that this may reflect the age of the patient and not the presence of a BRAF mutation (Trovisco et al. 2005). However, others have shown that PTC with BRAF mutation displays an increased incidence of locoregional recurrence and a decreased response to radioactive iodine. (Nikiforova et al. 2003; Namba et al. 2003; Xing et al. 2005; Vasko et al. 2005).

BRAF mutations in PTC are probably involved in tumor initiation, as they are found in microscopic PTC (microcarcinomas: e.g. PTC smaller than 10 mm diameter) (Xing et al. 2005). Notably, BRAFV600E is required to maintain and promote thyroid cancer progression (Nucera et al. 2010). Mechanistically, how BRAFV600E mutation leads to an aggressive PTC phenotype is still poorly understood; these mechanisms may include aberrant methylation of important tumor suppressor genes (i.e. TIMP-3), up-regulation of metallo-proteases-2, -3, -9 and -13 (MMP-2, -3, -9 and -13) (Mesa et al. 2006; Frasca et al. 2008), reduced expression of thyroperoxidase (TPO) and sodium iodide symporter (NIS) (important for iodine metabolism and iodine cell uptake) (Romei et al. 2008), and increased chromosomal instability (Mitsutake et al. 2005).

Thus, the high prevalence and specificity of BRAFV600E for PTC and ATC has led to great enthusiasm about the potential clinical utility of this mutation as a novel prognostic molecular marker and as an effective target for the treatment of PTC (Chiloeches et al. 2006; Espinosa et al. 2007; Groussin and Fagin 2007; Lee et al. 2007; Trovisco et al. 2006; Wojciechowska and Lewinski 2006; Xing 2006).

1.7 Kinases in Cancer

Kinases and cancer have been intimately linked for 30 years, since the first cellular oncogene, SRC, was discovered by Harold Varmus and Michael Bishop in 1975, and later found to encode a tyrosine kinase. Over 500 protein kinases have been identified to date, including many involved in cellular signaling pathways whose dysregulation has been implicated in cancer (Manning et al. 2002; Krause and Van Etten 2005). Indeed, many kinases have been found to be intimately involved in the processes leading to tumour cell proliferation and survival (**Table 2**). Point mutation, gene amplification or translocation, these oncogenic kinases have become impervious to normal regulatory mechanisms. Mutated kinases have transforming capacity and are therefore considered to be oncogenic.

Table 2: Kinases implicated in human cancer

	Kinase	Tumor Type	Oncogenic Alteration
RTK	EGFR	Breast, lung, glioma	Extracellular domain deletions & point mutations (L858R, G719S, & deletions)
	HER2/ErbB2	Breast, ovarian, colon, lung, gastric	Overexpression
	IGF-1R	Colon, pancreatic, breast, ovarian, MM	Overexpression
	PDGFR-α	Glioma, glioblastoma, ovarian, HES	Overexpression & translocation
	PDGFR-β	CMML, glioma, DFSP	Translocation
	c-Kit	GIST, seminoma, mastocytosis	Point mutations (D816V)
	Flt-4, Flt3	AML	Internal tandem duplication
	FGFR1	CML, Stem cell myeloproliferative disorder	Translocation (BCR-, FOP-, ZNF198-, CEP110-)
	FGFR3	MM	Translocation & point mutations (S249C)
	FGFR4	Breast, ovary	Overexpression
	c-Met	Glioblastoma, Colorectal, Hepatocellular carcinoma, renal carcinoma, HNSCC metastases	Overexpression, translocation (Tpr-), & point mutations (Y1253D)
	RON	Colon, hepatocellular carcinoma	Overexpression
	RET	papillary thyroid carcinoma, MEN2A, MEN2B, FMTC & sporadic MTC	Translocations & point mutations
	ALK	Anaplastic large cell lymphoma, lung, neuroblastoma	Translocations (NPM-, EML4); point mutations
CTK	c-SRC	Lung, colon, breast & prostate	Overexpression, C-terminal truncation
	c-YES	Lung, colon, breast & prostate	Overexpression
	Abl	CML	Translocation (Bcr-)
	JAK-2	CML, T-ALL, solid	Translocation (Tel-), point mutation (V617F)
S/T Kinase	Akt	Multiple	Overexpression
	ATM	Ataxia telangectasia	Point mutations
	Aurora A & B	Multiple	Overexpression
	CDKs	Multiple	Overexpression
	mTOR	Multiple	Overexpression
	PKCi	Non-small cell lung, ovarian	Overexpression
	PLK	Multiple	Overexpression
	BRAF	Melanoma, thyroid, colon	Point mutation (V600E)
	S6K	Multiple	Overexpression
	STK11/LKB1	Peutz-Jeghers syndrome	Point mutations
LK	PI3K	Prostate, colorectal, breast	Overexpression, point mutations (H1047R)

Table 2: Summary of kinases mutated in cancer (modified from Zhang et al. 2009). RTK: receptor tyrosine kinase, CTK: cytoplasmic tyrosine kinase, S/T Kinase: serine/threonine kinase, LK: lipid kinase. MM: Multiple Myeloma; HES: Hypereosinophilic Syndrome; CMML: Chronic Myelomonocytic Leukemias; DFSP: Dermatofibrosarcoma Protuberans; AML: Acute Myelogenous Leukemia; GIST: Gastrointestinal Stromal tumor; HNSCC: Head and neck squamous cell carcinoma; MEN2A: Multiple Endocrine Neoplasia type 2A Syndrome; MEN2B: Multiple Endocrine Neoplasia type 2B Syndrome; FMTC: Familial Medullary Thyroid Carcinoma; CML: Chronic Myelogenous Leukemia; T-ALL: T cell Acute Lymphoblastic Leukemia.

Intensive efforts to survey the kinome across a broad range of tumour types for mutations have uncovered a large number of kinases bearing mutations, which are currently in the process of being functionally characterized (Futreal et al. 2004; Forbes et al. 2005).

1.8 Kinase targeting in cancer treatment

The constitutive activity of kinases in cancer makes them essential for survival and/or proliferation of cancer cells. This so-called oncogene addiction renders the cancer cell susceptible to the appropriate therapeutic tool able to intercept kinase function (Weinstein and Joe 2006).

The interest of kinase therapeutic targeting in cancer extends further beyond kinases that are directly mutated in cancer cells. Indeed, besides the presence of mutations, there are other mechanisms through which kinases can be essential for tumor initiation or growth. In some cases, although not oncogenic and not mutated in cancer, some kinases are preferentially required for the survival and/or proliferation of the cancer cells. Thus, inhibition of the kinase results in a synthetic-lethal phenotype when paired with specific mutations present in cancer cells (Kaelin 2005). In some cases, such an essential role is explained by the fact that these kinases function in key signaling pathways downstream of transforming oncogenes. Examples include MEK1 and MEK2 (also known as MAP2K1 and MAP2K2), which are located in the MAPK pathway and whose inhibition might be synthetic lethal with mutation in BRAF (Wang et al. 2007), or mTOR (also known as FRAP1), which is located in the PI3K–AKT signaling system (Faivre et al. 2006) and whose inhibition might be synthetic lethal with mutations in PI3K or AKT. Other examples of this class of targets include kinases that are required to sustain rapid proliferation and/or survival in the presence of abnormal ploidy, such as the cyclin-dependent kinases (CDKs), which regulate cell cycle transition, Aurora kinases, which are essential for accurately dividing chromosomes, and Polo-like kinases, which are important during both mitosis and cytokinesis (Malumbres and Barbacid 2007).

Finally, a third class of kinase targets are those expressed in the tumour stroma rather than tumor cells and are required for tumour formation and maintenance. Examples of this category are represented by the vascular endothelial growth factor receptor (VEGFR) and the fibroblast growth factor receptor (FGFR), which are important in developing and sustaining tumour blood supply (Kerbel 2008).

There are several possibilities to target therapeutically kinases in cancer; these include monoclonal antibodies that can bind to the extracellular domain of the kinase, compounds able to favour the proteolytic degradation of the kinase and small molecule protein kinase inhibitors (PKI). Monoclonal antibodies have had a great success in the case of specific kinases and cancer types, like for instance HER2-directed trastuzumab in breast cancer and epidermal growth factor receptor (EGFR)-directed cetuximab in colon cancer (Baselga 2006). In this dissertation, we have focused in particular on small molecule kinase inhibitors.

1.9 Small molecule protein kinase inhibitors (PKI)

Protein kinases are defined by their ability to catalyse the transfer of the terminal phosphate of ATP to substrates that usually contain a serine, threonine or tyrosine residue. They typically share a conserved arrangement of secondary structure elements that are arranged into 12 subdomains that fold into a bi-lobed catalytic core with ATP binding in a deep cleft located between the 2 lobes (Johnson et al. 1998; Manning et al. 2002; Traxler and Furet 1999). ATP binds in the cleft with the adenine ring forming hydrogen bonds with the kinase ‘hinge’ — the segment that connects the amino- and carboxy-terminal kinase domains. The ribose and triphosphate groups of ATP bind in a hydrophilic channel extending to the substrate-binding site that features conserved residues that are essential to catalysis. All kinases have a conserved activation loop, which is important in regulating kinase activity and is marked by conserved DFG and APE motifs at the start and end of the loop, respectively. The activation loop can assume a large number of conformations with the extremes being a conformer that is catalytically competent and usually phosphorylated, and an ‘inactive’ conformer in which the activation loop blocks the substrate binding site.

Based on this structure, protein kinase inhibitors (PKI) have been designed to fit within the kinase domain and block the function of the kinase. Most PKIs discovered to date are ATP competitive and present one to three hydrogen bonds to the amino acids located in the hinge region of the target kinase, thereby mimicking the hydrogen bonds that are normally formed by the adenine ring of ATP (Traxler and Furet 1999; Liu and Gray 2006) (**Figure 8**). The majority of PKIs do not exploit the ribose-binding site (an exception being AZD0530, a novel Src and Abl dual family kinase inhibitor) or the triphosphate-binding site of ATP. Overall, protein kinase inhibitors (PKI) are classified in: class1, class 2, allosteric and covalent inhibitors.

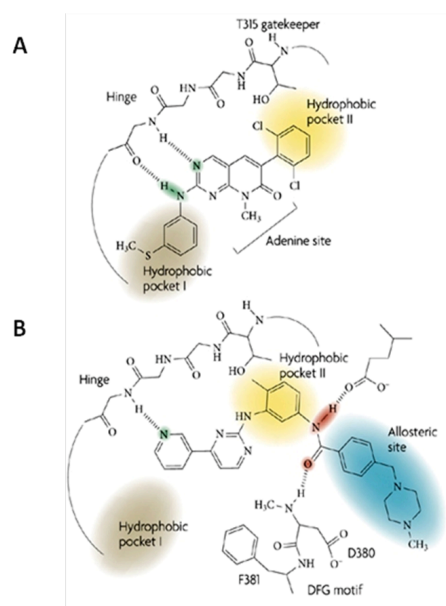


Figure 8: Class 1 and class 2 inhibitors–protein kinase interactions. A. The chemical structure depicts hydrophobic regions I and II of ABL1 (shaded beige and yellow respectively); hydrogen bonds between the class 1 kinase inhibitor (inhibitor atoms engaged in hydrogen bonds to hinge or to allosteric site in red) and ABL1 are indicated by dashed lines. B. The DFG-out conformation of the activation loop of ABL1 (dark blue) with the type 2 inhibitor imatinib. The allosteric pocket exposed in the DFG-out conformation is indicated by the blue shaded area (modified from Zhang et al. 2009).

1.9.1 Type 1 inhibitors

This class includes the majority of ATP-competitive inhibitors and recognizes the so-called active conformation of the kinase (DFG-in) a conformation otherwise conducive to phosphotransfer (Liu and Gray 2006) (**Figure 8A**). Most of available kinase inhibitors belong to class 1; this may be a consequence of the fact that most compounds were discovered using enzymatic assays in which kinases were introduced in their active conformation and also of the fact that many kinase inhibitors have been synthesized to mimic ATP. Type 1 inhibitors typically consist of a heterocyclic ring system that occupies the purine-binding site, where it serves as a scaffold for side chains that occupy the adjacent hydrophobic regions I and II.

1.9.2 Type 2 inhibitors

Type 2 kinase inhibitors recognize the inactive conformation of the kinase (**Figure 8B**). The conformation that is recognized by type 2 inhibitors is referred to as DFG-out, owing to the rearrangement of this motif. Movement of the activation loop to the DFG-out conformation exposes an additional hydrophobic binding site directly adjacent to the ATP binding site. The original discovery that inhibitors such as imatinib and sorafenib bind in the type 2 conformation was serendipitous, but subsequent analysis of multiple type 2 kinase inhibitor co-crystal structures has revealed that all share a similar pharmacophore and exploit a conserved set of hydrogen bonds. Examples of type 2 inhibitors that are approved by the US Food and Drug Administration include the ABL1, KIT and platelet-derived growth factor receptor (PDGFR) inhibitors imatinib and nilotinib (Manley et al. 2005), and the KIT, PDGFR and RAF inhibitor sorafenib (Wan et al. 2004). The inhibitor stabilized conformational rearrangement of the activation loop observed in type 2 kinase inhibitor co-crystal structures demonstrates that the kinase active site is highly mobile and can remodel to accommodate a variety of inhibitors (Liu and Gray 2006).

1.9.3 Allosteric inhibitors

The third class of compounds binds outside the ATP-binding site at an allosteric site and modulates kinase activity in an allosteric manner. Inhibitors belonging to this category exhibit the highest degree of kinase selectivity because they exploit binding sites and regulatory mechanisms that are unique to a particular kinase. The most well characterized allosteric kinase inhibitor is CI-1040, which inhibits MEK1 and MEK2 by occupying a pocket adjacent to the ATP binding site (Ohren et al. 2004) (**Figure 9**). Other examples include: GNF2, which binds to the myristate binding site of BCR–ABL1 (Adrián et al. 2006); the pleckstrin homology domain-dependent Akt inhibitor Akt-I-1 (Barnett et al. 2005; Lindsley et al. 2005); the IKK (inhibitor of nuclear factor- κ B kinase) inhibitor BMS-345541 (McIntyre et al. 2003).

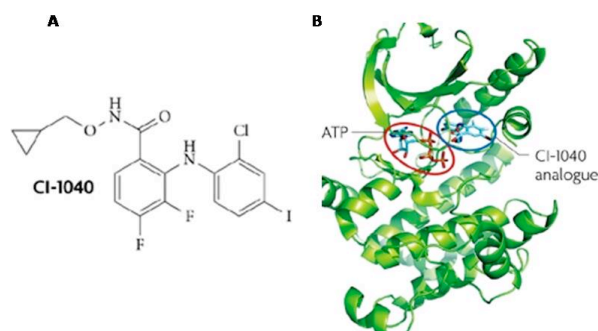


Figure 9: Allosteric kinase activity modulators. CI-1040 (indicated by a blue circle) binds MEK1 (green ribbon) immediately adjacent to the ATP binding site (indicated by red circle) (modified from Zhang et al. 2009).

More allosteric inhibitors are likely to be uncovered in the future, as a greater emphasis is placed on cell-based assays in which kinases are inhibited in their native context. This approach presents the advantage of allowing compounds to be identified that may require an accessory protein for function. For example, the requirement of the mTOR inhibitor rapamycin for the intracellular protein FKBP1A would not have been discovered in a biochemical mTOR assay.

1.9.4 Covalent inhibitors

A fourth class includes kinase inhibitors that are capable of forming an irreversible, covalent bond to the kinase active site, most frequently by reacting with a nucleophilic cysteine residue (Kwak et al. 2005). The clinically most advanced covalent inhibitors are the irreversible kinase inhibitors of the epidermal growth factor receptor (EGFR), HKI-272 (Rabindran et al. 2004) and CL-387785 (Kobayashi et al. 2005). These two compounds were developed to target a relatively specific cysteine residue located at the lip of the ATP binding site (Fry et al. 1994).

1.10 Mechanisms of resistance to kinase inhibitors (PKI)

Two major mechanisms have been envisaged to allow cancer cells to escape treatment with PKI. Target amplification has been also reported as in the case of BCR–ABL1 in CML patients (le Coutre et al. 2000). Owing to the rapid proliferation of cancer cells, the acquisition of mutations conferring drug resistance has become a recurring theme in the clinic. Indeed, resistance as a result of kinase mutations has been documented for inhibitors of BCR–ABL1, EGFR, FLT3, KIT and PDGFR (Kobayashi et al. 2005; Roumiantsev et al. 2002; Fletcher and Rubin 2007; Cools et al. 2004). To date the most extensive clinical and laboratory characterization of resistance-causing mutations has been performed for BCR–ABL1 in the context of imatinib and second-generation inhibitors.

Alternatively, cancer cells can escape treatment by switching to an alternative-signaling pathway that is not inhibited by the PKI (Stommel et al. 2007). This is the case as an example of the activation of hepatocyte growth factor receptor (HGFR/MET) in the acquisition of resistance to EGFR kinase inhibitors in lung cancer (Arteaga 2007).

Additionally, it has been shown in several haematological tumours that quiescent stem cells are refractory to tyrosine kinase inhibitors, and these cell populations are probably also involved in resistance mechanisms (Copland et al. 2006).

As far as mutations in the target, resistance conferred by mutation at the gatekeeper residue—so called because the size of the amino acid side chain at this position determines the relative accessibility of a hydrophobic pocket located adjacent to the ATP binding site (hydrophobic pocket II) —appears to be a common mechanism for a variety of kinases. Access to this pocket is important to many PKIs because hydrophobic interactions in this site are crucial for the binding affinity of the inhibitor. For example, the most recalcitrant of the BCR–ABL1 mutants is T315I, which harbours a mutation in the gatekeeper residue. Although the gatekeeper residue often comes in close contact with type 1 and type 2 ATP binding site inhibitors, it typically does not interact with ATP (Liu and Gray 2006). Consequently, mutation of the gatekeeper residue generally causes little or no change in kinase activity but has the potential to confer inhibitor resistance through a variety of biochemical mechanisms. Likewise, mutation of the EGFR T790 residue to methionine induces resistance to the quinazoline-based inhibitors gefitinib and erlotinib (Pao et al. 2005) by increasing the affinity for ATP, which effectively weakens the affinity of ATP-competitive inhibitors. The gatekeeper mutation G697R in FLT3 induces resistance to the type 1 staurosporine derivative PKC412 (Cools et al. 2004). The gate-keeper mutation in RET (V804M/L) induces resistance to vandetanib (Carlomagno et al. 2004). There is currently debate as to whether the gatekeeper mutation is pre-existing or acquired following inhibitor treatment, though most evidences suggest the mutation pre-exists in a

minority of cancer cells, and it is then selected upon PKI treatment. Whatever the case, the gatekeeper mutants of BCR–ABL1 (T315I), PDGFR α (T674I), EGFR (T790M) and KIT (T670I) are the most frequently reported mutants for each of these kinases. This suggests that of design inhibitors that can overcome this mutation are urgently needed.

1.11 Kinase inhibitors in thyroid cancer: RET inhibitors

The management of patients with thyroid cancer depends on the histological subtype and stage of the tumour. Among thyroid cancers, a great majority of WDTC patients can be cured with the conventional treatment regimen composed of surgery, radioactive iodine and thyroid hormone therapy (Cooper et al. 2009). Less than 10% of WDTC patients present with or ultimately develop distant metastases (Durante et al. 2006; Elisei et al. 2010; Lee and Soh 2010). Around 40-50% of these subjects are refractory to radioactive iodine treatment (Durante et al. 2006; Schlumberger et al. 1996) and have a 10-year survival rate of 10-20%. In radioactive iodine resistant subjects, conventional chemotherapy response rates are low and long term cure is rare (Sherman 2010). On the other hand, for patients with MTC, which comprises around 5% of thyroid cancers, the only curable treatment choice is surgery (Schlumberger et al. 2008). For the treatment of ATC, which is the least common histologic type of thyroid cancer, surgery, radiotherapy and chemotherapy have been used with poor results (Smallridge et al. 2009).

Thus, use of PKI has been considered for the treatment of MTC and radioiodine-refractory WDTC and ATC. Considering the role of growth factor tyrosine kinase signaling pathway in the pathogenesis of thyroid cancers, PKIs targeting this pathway, might be a rational choice for treatment (**Table 3**). Randomised clinical trials for several agents are underway and may lead to approval of PKIs for thyroid cancer. Some of these inhibitors target RET and therefore might be useful in those cancer types, primarily MTC, in which RET is involved. These agents are multitargeted and able to inhibit several kinases besides RET. Combined action on several targets may have the advantage of blocking simultaneously pathways active in both tumor parenchyma and stroma (Knight et al. 2010).

ZD6474 (vandetanib) is an anilinoquinazoline TKI (Wedge et al. 2002; Herbst et al. 2007). ZD6474 docks in the ATP-binding pocket of the RET kinase (Knowles et al. 2006) and inhibits RET kinase with an inhibitory concentration 50 (IC₅₀) of 100-130 nM (Carlomagno et al. 2002; Herbst et al. 2007). ZD6474 reduced proliferation and tumorigenicity of fibroblasts expressing RET-derived oncogenes (Carlomagno et al. 2002); it was also effective in a transplanted mouse model of human MTC (Johanson et al. 2007) and in a *Drosophila* model of RET-mediated tumorigenesis (Vidal et al. 2005). ZD6474 shares with certain other RET TKIs (sorafenib, sunitinib, XL-184) the

capability of targeting vascular endothelial growth factor receptor type 2 (VEGFR2/KDR) (IC_{50} = 38-40 nM), VEGFR3 (Flt-4) (IC_{50} = 110-260 nM) and VEGFR1 (Flt-1) (150 - >1,000 nM) (Wedge et al. 2002; Bianco et al. 2008). In addition, ZD6474 targets the epidermal growth factor receptor (EGFR) (IC_{50} = 43-500 nM), a kinase that plays a prominent role in epithelial cell malignancies (Wedge et al. 2002; Ciardiello et al. 2003; Bianco et al. 2008). ZD6474 was studied in a Phase III clinical trial in MTC and found to be able to prolong progression free survival of MTC patients with respect to placebo. Based on these data vandetanib has been FDA approved for the treatment of locally advanced or metastatic MTC (Wells et al. 2011).

Table 3: Tyrosine Kinase inhibitors in current trials for thyroid cancer and their targets.

Drug	Targeted tyrosine kinase
Axitinib	VEGFR1-3, PDGFR, c-Kit
Lenvatinib (E7080)	VEGFR1-3, FGFR1-4, RET, c-Kit, PDGFR
Motesanib	VEGFR1-3, PDGFR, c-Kit, RET
Pazopanib	VEGFR, PDGFR, c-Kit
Sorafenib	VEGFR1-3, PDGFR, RET, RAF, c-Kit
Sunitinib	PDGFR, VEGFR1-3, c-Kit, RET, CSF1R, FLT3
Vandetanib (ZD6474)	VEGFR2-3, RET, EGFR

Abbreviations: CSF1R, macrophage colony-stimulating factor 1 receptor; EGFR, Epidermal growth factor receptor; FGFR, Fibroblast growth factor receptor; FLT3, FL cytokine receptor; HGFR, Hepatocyte growth factor receptor; PDGFR, Platelet-derived growth factor receptor; VEGFR, Vascular endothelial growth factor receptor.

1.12 Kinase inhibitors in thyroid cancer: PLX4032 (Vemurafenib)

PLX4032 (RG7204, Vemurafenib) is a potent inhibitor of BRAF with the V600E mutation. PLX4032 abrogates signaling through the MAP kinase pathway, and blocks proliferation of cells carrying BRAF with the V600E mutation (Tsai et al. 2008). PLX4032 belongs to a family of mutant BRAF kinase inhibitors discovered using a scaffold-based drug design approach. The crystallography guided approach allowed optimization of a compound with modest preference for the mutated form of BRAF in comparison to wild-type BRAF (**Figure 10**). Preclinical studies showed that BRAF mutant melanoma cell strains were highly sensitive to PLX4032 with IC_{50} in the nM range (60–450 nM), whereas BRAF wild-type cells were resistant, with IC_{50} 2.4 μ M or above (Halaban et al. 2010). The biochemical selectivity of vemurafenib (PLX4032) translates to cellular selectivity: potent inhibition of ERK phosphorylation and cell proliferation occurs exclusively in BRAF-mutant cell lines (Yang et al. 2010).

PLX4032 was co-crystallized with a protein construct that contained the kinase domain of BRAF (V600E). PLX4032 binds in the active site of one of the protomers in the non-crystallographic-symmetry related dimer (**Figure 10**). In addition, PLX4032-binding causes an outward shift in the regulatory α_C helix. The apo-protomer displays the DFG-in conformation with the activation loop locked away from the ATP-binding site by a salt-bridge between Glu 600 and Lys 507 (**Figure 10**) (Bollag et al. 2010).

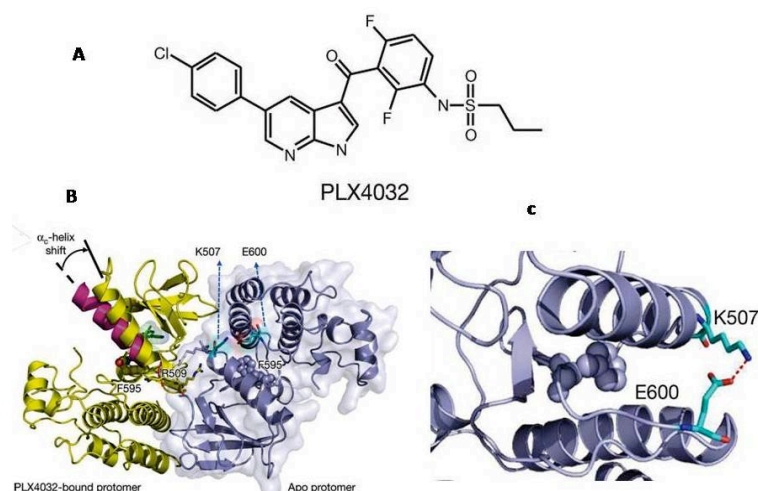


Fig. 10: PLX4032 (Vemurafenib). **A.** Chemical structure of PLX4032. **B.** Structure of the asymmetric dimer of BRAF (V600E) is shown with the PLX4032-protomer bound to PLX4032 coloured yellow. The surface outline of the other protomer (blue) is shown lightly shaded. Highlighted residues are R509 to reflect its role in anchoring the dimer and F595 to show that both protomers are in the DFG-in state. The α_C -helix shown in magenta is overlaid on the PLX4032-bound protomer to show its typical configuration in an unoccupied protomer; the binding of PLX4032 causes a shift of the α_C -helix as noted by the arrow. **C.** Magnified view of the salt bridge between Lys 507 and Glu 600 that helps prevent compound binding to the apo protomer (modified from Bollag et al. 2010).

Clinically, the efficacy and safety of vemurafenib in patients with treatment-naïve unresectable or metastatic melanoma was studied in a randomized open-label phase III trial. Analysis of the data available up to this point, showed that after 6 months, overall survival for patients receiving vemurafenib was 84%, compared to 64% for patients receiving dacarbazine. The estimated median progression-free survival (PFS) was 5.3 months for patients receiving vemurafenib, compared to 1.6 months for patients receiving dacarbazine (Chapman et al. 2011). Based on these data, in August 2011, vemurafenib was approved by the US Food and Drug Administration (FDA)

for the treatment of patients with unresectable or metastatic melanoma with the BRAFV600E mutation.

1.13 Resistance to PLX4032

Current translational research is focusing on understanding mechanisms of resistance to vemurafenib and the development of rational combination regimens. Different PLX4032-resistant melanoma cell lines showed different patterns of ERK and MEK activation, indicating that more than one resistance mechanism was involved, and this was confirmed using gene expression profiling.

Some cell lines showed significant increases in the activation of MAPK pathway in some cases explained by the development of mutations in NRAS (**Figure 11**). A second subset of cell lines showed high levels of the growth factor receptor PDGFR β . These cells have low levels of MAPK pathway activity, indicating that tumour cell survival is mediated via switch to an alternative signal transduction pathway (**Figure 11**) (Nazarian et al. 2010).

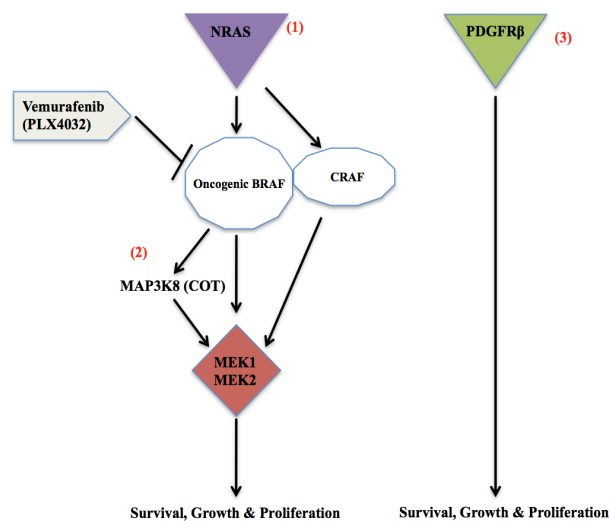


Fig. 11: Resistance to vemurafenib can be mediated by MAPK re-activation, for instance due to NRAS mutation (1) or COT activation (2) or to shift to an alternative signaling pathway, like for instance activation of PDGFR (3).

In another study, a large group of researchers led by Levi Garraway and Cory Johannessen at the Broad Institute of Harvard and MIT expressed a panel of about 600 human kinases in PLX4032 sensitive melanoma cell lines and treated these with the BRAF kinase inhibitor to identify kinases that could

confer resistance to the drug. Two kinases, COT and CRAF – both of which are members of the Ser/Thr MAP kinase kinase kinase family – were highlighted in particular as conferring the most significant resistance in multiple cell lines. These kinases are involved in the MAPK pathway, and their ability to confer resistance suggests that this is arising through re-activation of this pathway (Johannessen et al. 2010) (**Figure 11**).

Taken together, these studies indicate that resistance to this promising drug can arise through a variety of mechanisms, some involving downstream kinases in the MAPK pathway but none involving secondary mutations in the BRAF gene.

2. AIMS OF THE STUDY

Mutations in RET (V804 and Y806) causing resistance to RET PKI have been described. Mechanisms of thyroid cancer cell resistance to BRAF PKI are still unknown. Aims of this study were:

1. To identify RET PKI able to overcome resistance mediated by V804 and Y806 RET mutations;
2. To explore mechanisms of resistance to BRAF PKI (vemurafenib) and devise strategies to overcome such resistance.

In addition, this study has been focused on the role of beta-catenin signaling pathway in thyroid carcinogenesis and on the functional characterization of a novel RET point mutation found in MTC. The resulting manuscripts are attached at the end of this Dissertation.

3. MATERIALS & METHODS

3.1 Compounds

Ponatinib (AP24534) was provided by ARIAD Pharmaceuticals Inc, (Cambridge, MA, USA). For *in vitro* experiments, Ponatinib was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mM. For *in vivo* experiments, it was dissolved in Sodium Citrate, pH - 2.7 and stored at room temperature. Vemurafenib (PLX4032) was provided by Roche Pharmaceuticals (Nutley, NJ, USA). PLX4032 was dissolved in DMSO at a concentration of 50 mM and stored at -80°C.

3.2 Cell Culture

HEK293 cells were from American Type Culture Collection (ATCC) and were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 units/ml penicillin-streptomycin (GIBCO). Transient transfections were carried out with the lipofectamine reagent according to manufacturer's instructions (GIBCO). NIH3T3 mouse fibroblasts stably expressing the oncogenic RET mutants, associated to sporadic and familial MTC, were grown in DMEM supplemented with 5% Calf Serum. TT cells were from American Type Culture Collection. TT was derived from the primary tumor of an apparently sporadic MTC (Leong et al. 1981). TT harbor a cysteine 634 to tryptophan (C634W) exon 11 RET mutation (Carlomagno et al. 1995) as well as a tandem duplication of the mutated RET allele (Huang et al. 2003). MZ-CRC-1 cells were kindly provided by Robert F. Gagel. MZ-CRC-1 was derived from a malignant pleural effusion from a patient with a metastatic MTC (Cooley et al. 1995). MZ-CRC-1 cells bear a heterozygous (ATG to ACG) transition in RET exon 16 resulting in the MEN2B-associated substitution of threonine 918 for methionine (M918T). TT cells were grown in RPMI 1640 supplemented with 16% fetal calf serum (GIBCO, Paisley, PA). MZ-CRC-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. All media were supplemented with 2 mM L-glutamine and 100 units/ml penicillin-streptomycin (GIBCO). Human papillary (TPC1, BCPAP) and anaplastic (BHT101, SW1736) thyroid cancer cells are described elsewhere and were grown in DMEM with 10% Fetal Calf Serum L-glutamine and penicillin/streptomycin (Salerno et al. 2011). NTHY (Nthy-ori 3-1) are normal human thyrocytes immortalized by the Large T of SV40. NTHY were grown in DMEM supplemented with 10% FBS, L-glutamine and penicillin/streptomycin. All the cells were SNP genotyped to ensure correct identity.

3.3 Cell Proliferation Assays

5×10^4 cells were plated in 35-mm dishes. The day after plating, the compounds or vehicle were added. Cells were counted in triplicate every day for four days and to estimate EC50 value, cells were counted after three days. For RNA silencing, the small inhibitor duplex RNAs (siRNA) (ON-target plus SMARTpool) siIGF1R were from Dharmacon (Lafayette, CO, USA). The siCONTROL Non-targeting Pool (#D-001206-13-05) was used as a negative control. Cells were transfected with 100 nM siRNAs using Dharmafect reagent following manufacturer's instructions. The day before transfection, the cells were plated in 35 mm dishes at 40% of confluence in RPMI supplemented with 4% FBS without antibiotics. Cells were harvested 48 hours after transfection.

3.4 Quantitative RT-PCR

For evaluation of mRNA expression levels, cell lines were grown to 70% confluency in DMEM with 10% FBS, and then total RNA was extracted with RNeasy mini kit (Qiagen) and subjected to on-column DNase digestion with the RNase-free DNase set (Qiagen) according to the manufacturer's instructions. Random-primed first strand cDNA was synthesized in a 50 μ l reaction volume starting from 2 μ g RNA by using the GeneAmp RNA PCR Core Kit (Applied Biosystems). PCR amplification was performed using the GeneAmp RNA PCR Core Kit system starting from 2.5 μ l of RT product in a reaction volume of 25 μ l according to the manufacturer's instructions. Primers were designed by using a software available at <http://www-genome.wi.mit.edu/cgi-bin/primer/primer3> www.cgi and synthesized by CEINGE (Naples, Italy). Quantitative (real-time) polymerase chain reactions (qRT-PCR) were performed by using the SYBR Green PCR Master mix (Applied Biosystems) in the iCycler apparatus (Bio-Rad). Amplification reactions (25 μ l final reaction volume) contained 200 nM of each primer, 3 mM MgCl₂, 300 μ M dNTPs, 1x SYBR Green PCR buffer, 0.1U/ μ l AmpliTaq Gold DNA Polymerase, 0.01U/ μ l Amp Erase, RNase-free water, and 2 μ l cDNA samples. To verify the absence of non-specific products, 80 cycles of melting (55°C for 10 sec) were performed. In all cases, the melting curve confirmed that a single product was generated. Amplification was monitored by measuring the increase in fluorescence caused by the SYBR-Green binding to double-stranded DNA. Fluorescent threshold values were measured in triplicate and fold changes were calculated by the $\Delta\Delta C_t$ formula: $2^{-(\text{sample 1 } \Delta C_t - \text{sample 2 } \Delta C_t)}$, where ΔC_t is the difference between the cycle threshold (Ct) of the mRNA of interest and the Ct of the β -actin mRNA used as housekeeping gene.

3.5 Protein Studies

Immunoblotting experiments were performed according to standard procedures. Briefly, cells were harvested in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM MgCl₂, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na₃VO₄, 10 µg of aprotinin/ml, 10 µg of leupeptin/ml) and clarified by centrifugation at 10,000 g. Protein concentration was estimated with a modified Bradford assay (Bio-Rad). Antigens were revealed by an enhanced chemiluminescence detection kit (ECL, Amersham Pharmacia Biotech). Signal intensity was quantified with the Phosphorimager (Typhoon 8600, Amersham Pharmacia Biotech) interfaced with the ImageQuant software. Anti-phospho-Shc (#Y317), which recognizes Shc proteins when phosphorylated at Y317, was from Upstate Biotechnology Inc. Anti-Shc (H-108) was from Santa Cruz Biotechnology. Anti-MAPK (#9101) and anti-phospho-MAPK (#9102), specific for p44/42MAPK (ERK1/2) phosphorylated at Thr202/Tyr204, were from Cell Signaling. Anti-phospho MEK1/2 (#9121), anti-MEK1/2 (#9122), anti-IGF1R, anti-phospho-IGF1R, anti-CRAF and anti-phospho-CRAF (serine 338) antibodies were from Cell Signaling. Anti-BRAF (sc-9002) was from Santa Cruz Biotechnology. Anti-phospho AKT (specific for phosphorylated serine 473 or threonine 308) and anti-AKT antibodies were from Cell Signaling Technology. Anti-RET is a polyclonal antibody raised against the tyrosine kinase protein fragment of human RET. Anti-pY905 and anti-pY1062 are phospho-specific affinity-purified polyclonal antibodies that recognize RET proteins phosphorylated at Y905 and Y1062, respectively. Monoclonal anti- α -tubulin was from Sigma Chemical Co. Secondary antibodies coupled to horseradish peroxidase were from Amersham Pharmacia Biotech.

3.6 Phospho-RTK Arrays

The relative levels of tyrosine phosphorylation of receptor tyrosine kinases (RTKs) were evaluated with a Human Phospho-RTK array kit (R&D Systems, Inc.). Briefly, harvested cells were lysed in NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 137 mM NaCl, 10% Glycerol, 2 mM EDTA, 1 mM Sodium Orthovanadate, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin) and centrifuged at 14,000Xg for 5 minutes at 4°C. For each array, 500 µg of lysate were used. Each array contained 42 different anti-RTK antibodies and six controls printed in duplicate. Non-specific binding was blocked for 1 hour at room temperature on a rocking platform shaker. The lysates were diluted in Array Buffer 1 (provided with the kit) and added to each array for overnight incubation at 4°C. Freshly diluted detection antibody was added to the arrays for 2 hours of room temperature incubation. Phosphorylation levels were detected by chemiluminescence (ECL; Amersham Pharmacia Biotech).

3.7 In vitro kinase assay

The *in vitro* RET auto-phosphorylation assay was performed as described elsewhere (Carlomagno et al. 2006). Briefly, subconfluent cells were solubilized in lysis buffer without phosphatase inhibitors. Then, 50 µg of proteins were immunoprecipitated with anti-RET; immunocomplexes were recovered with protein A sepharose beads, washed 5 times with kinase buffer and incubated 20 min at room temperature in kinase buffer containing 2.5 µCi [γ -³²P] ATP and unlabelled ATP to a final concentration of 20 µM. Samples were separated by SDS-PAGE. Gels were dried and exposed to autoradiography. Signal intensity was analyzed at the Phosphorimager (Typhoon 8600) interfaced with the ImageQuant software.

3.8 Chemoinvasion Assay

In vitro invasiveness through Matrigel was assayed using transwell cell culture chambers. Briefly, confluent cell monolayers were harvested with trypsin/EDTA and centrifuged at 800Xg for 10 minutes. The cell suspension (1X10⁵ cells/well) was added to the upper chamber of transwells on pre-hydrated polycarbonate membrane filter of 8 µm pore size (Costar) coated with 35µg Matrigel (Collaborative Research Inc.). The lower chamber was filled with complete medium. Cell dishes were incubated at 37 °C in 5% CO₂ for 24h. Non-migrating cells on the upper side of the filter were wiped-off and migrating cells on the reverse side of the filter were stained with 0.1% crystal violet in 20% methanol for 15 minutes, counted and photographed.

3.9 Wound Healing Assay

A wound was inflicted on the confluent monolayer cells by scraping a gap using a micropipette tip. Photographs were taken at 100X magnification using phase-contrast microscopy immediately after wound incision and 12 hours later to assess whether cells had migrated into the wound.

3.10 Statistical Analysis

Two-tailed paired Student's t test (normal distributions and equal variances) was used for statistical analysis. Differences were significant when $P < 0.05$. Statistical analysis to estimate EC50 value was performed using the Graph Pad Prism and InStat software program (version 3.06.3, San Diego, CA, USA).

4. RESULTS

4.1 PONATINIB IS A NOVEL RET TKI ABLE TO OVERCOME RET GATE-KEEPER MUTATION MEDIATED RESISTANCE

4.1.1 Ponatinib efficiently inhibits RET kinase function.

Several RET kinase inhibitors (PKI) have been identified and one of them, vandetanib (ZD6474) was recently approved for the treatment of medullary thyroid carcinoma (MTC) (Santoro et al. 2006; Wells et al. 2009; Sherman 2011; Wells et al. 2011). However, RET mutations have been proved to mediate resistance to vandetanib. Indeed, we have previously reported that RET mutants in the V804 residue, naturally occurring in MTC patients, are refractory to inhibition by vandetanib and by pyrazolo-pyrimidines of the PP series (Carlomagno et al. 2004). At the structural level, this was caused by the particular position that V804 occupies in the RET kinase domain; indeed, V804 is the gate-keeper position, corresponding to T315 in ABL, at the entrance (gate) of the RET ATP binding pocket. Therefore, its replacement with bulky residues such as methionine or leucine, as it occurs in these mutants, impairs drug binding (Knowles et al. 2006). Also mutations targeting nearby residue Y806 strongly impair drug binding to the RET kinase (Carlomagno et al. 2009). Both V804 (to leucine or methionine) and, though very rarely, Y806 (to cysteine) mutations have been found in patients affected by MTC, thus raising the possibility that they may mediate a primary resistance to the treatment. It may also be envisaged that, should these mutations occur during treatment, they can also mediate a secondary (acquired) resistance. Therefore, it is important to isolate novel RET PKI that may overcome such resistance mechanisms.

Ponatinib (AP24534) is a novel multitargeted kinase inhibitor that potently inhibits native and mutant BCR-ABL at clinically achievable drug levels. Ponatinib also has in vitro inhibitory activity against a discrete set of kinases, including FLT3, KIT, fibroblast growth factor receptor 1 (FGFR1), and platelet derived growth factor receptor α (PDGFR α) (Gozgit et al. 2011; Zhou et al. 2011; Huang et al. 2010; O'Hare et al. 2009). Importantly, ponatinib (AP24534) inhibits both native and mutant BCR-ABL, including T315I mutant in the gate-keeper residue (corresponding to RET V804).

Thus, we sought to check whether ponatinib had activity against RET and its oncogenic variants, including those causing resistance to other PKIs. We initially studied whether ponatinib (AP24534) was able to inhibit *in vitro* RET enzymatic activity (Carlomagno et al. 2006). To this aim, HEK293 cells were transiently transfected with myc-tagged RET/C634R, an oncogenic RET mutant associated to MTC (Wells et al. 2009). RET protein was immunoprecipitated at room temperature in kinase buffer containing 2.5 μCi [γ - ^{32}P] ATP. Samples were separated by SDS-PAGE; gels were dried and exposed to Phosphorimager to measure extent of RET protein auto-phosphorylation. The average results of three independent assays showed that IC₅₀ of ponatinib for RET/C634R was 25 nM (95% confidence intervals 23.15 to 28.77) (**Figure 12**).

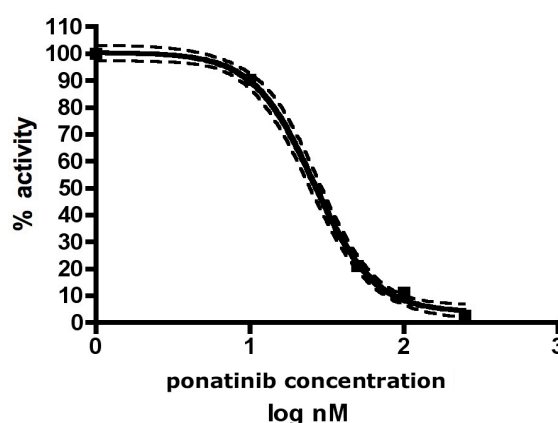


Fig. 12: Ponatinib inhibits *in vitro* RET autophosphorylation at nanomolar range doses. RET/C634R was immunoprecipitated and immunocomplexes were incubated in kinase buffer containing 2.5 μCi [γ - ^{32}P] ATP and different doses of ponatinib. Samples were separated by SDS-PAGE; gels were dried and exposed to Phosphorimager to measure extent of RET protein auto-phosphorylation. The average results of three independent assays are reported as % RET kinase activity with respect to vehicle-treated immunocomplexes by using the Graphpad software.

Then, we verified whether ponatinib had activity against vandetanib-resistant RET mutants. HEK293 cells were transfected with RET/V804M; RET protein was immunoprecipitated and immunocomplexes were incubated in kinase buffer containing 2.5 μCi [$\gamma\text{-}^{32}\text{P}$] ATP. Samples were separated by SDS-PAGE and dried gels were exposed to Phosphorimager. The average results of three independent assays showed that IC₅₀ of ponatinib for RET/V804M was 33 nM (95% confidence intervals 26.41 to 43.58), thus quite similar to that observed for the C634R mutant (**Figure 13**).

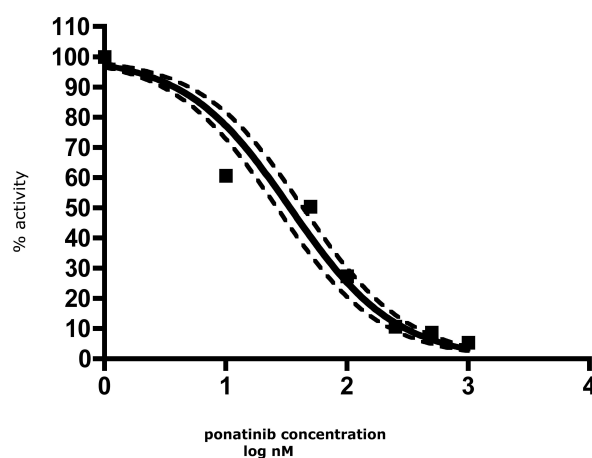


Fig. 13: Ponatinib inhibits in vitro autophosphorylation of RET mutant in the V804 gate-keeper position. RET/V804M was immunoprecipitated and immunocomplexes were incubated in kinase buffer containing 2.5 μCi [$\gamma\text{-}^{32}\text{P}$] ATP and different doses of ponatinib. Samples were separated by SDS-PAGE; gels were dried and exposed to Phosphorimager to measure extent of RET protein auto-phosphorylation. The average results of three independent assays are reported as % RET kinase activity with respect to vehicle-treated immunocomplexes by using the Graphpad software.

Overall, these findings prove that ponatinib is a potent RET kinase PKI, effective also against RET mutants in the gate-keeper residue.

4.1.2 Ponatinib efficiently inhibits RET oncoprotein phosphorylation in intact cells.

We sought to verify whether ponatinib activity against RET enzyme in vitro translated into an efficient reduction of phosphorylation and signaling of various RET mutants in intact cells. To this aim, RET point mutants depicted in **Figure 14** were selected (RET mutation database at www.arup.utah.edu/database/MEN2/MEN2_welcome.php or www.sanger.ac.uk/genetics/CGP/cosmic/). All these mutants have been associated to MTC familial cases (MEN2), sporadic or both (Waguespack et al. 2011). Of particular note are C634 and M918T mutants, those most frequently found in MEN2A or MEN2B, respectively. M918T is also the most prevalent RET mutant in sporadic MTC. Mutant A883 is found in the few MEN2B patients negative for M918T. V804 mutants (either to methionine or leucine), as well as Y806 mutant were also tested.

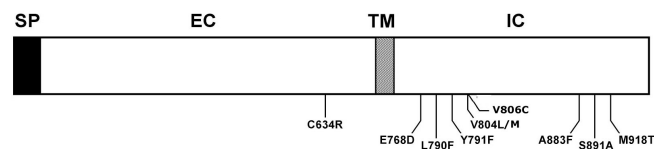


Fig. 14: Schematic representation of the RET mutants tested in this study.

HEK293 cells were transiently transfected with the various RET constructs and after 24 hours cells were treated with ponatinib at various doses for 60 min and harvested. RET phosphorylation was measured at two sites (Y905, the autocatalytic tyrosine and Y1062 the multidocking site) by immunoblot with phospho-specific antibodies (Salvatore et al. 2000). Phosphorylation of SHC, a RET substrate, was studied as well (Borrello et al. 1994). Though with some variability, ponatinib IC₅₀ for the phosphorylation of the various RET mutants was confirmed to be 10-50 nM (**Figures 15-24**). Again, importantly, ponatinib was effective (though slightly less than the other mutants) also against RET mutants in V804, the gatekeeper residue and in the nearby Y806 residue. In these cases, ponatinib IC₅₀ was about 50 nM. All together, these data indicate that ponatinib is a potent inhibitor of a large set of oncogenic RET mutants, including those bearing mutations in the gate-keeper residue.

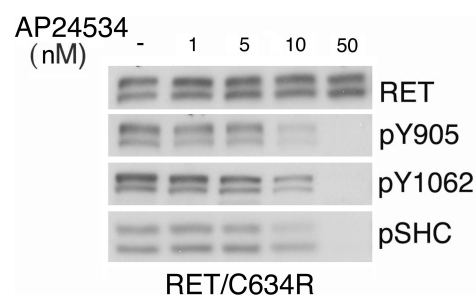


Fig. 15: Ponatinib inhibits in vivo phosphorylation of RET mutant in C634 position. HEK293 cells transiently transfected with indicated RET mutant were treated with indicated doses of compound for 60 min. Proteins were harvested and RET and SHC phosphorylation assessed by immunoblot. Total RET levels are shown for normalization.

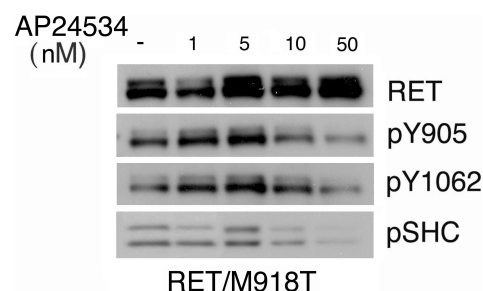


Fig. 16: Ponatinib inhibits in vivo phosphorylation of RET mutant in M918 position. For a description, see legend to Figure 15

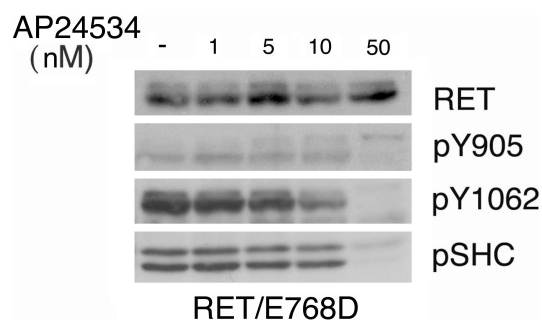


Fig. 17: Ponatinib inhibits in vivo phosphorylation of RET mutant in E768 position. For a description, see legend to Figure 15

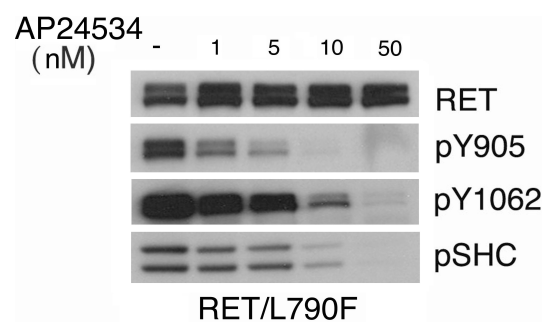


Fig. 18: Ponatinib inhibits in vivo phosphorylation of RET mutant in L790 position. For a description, see legend to Figure 15

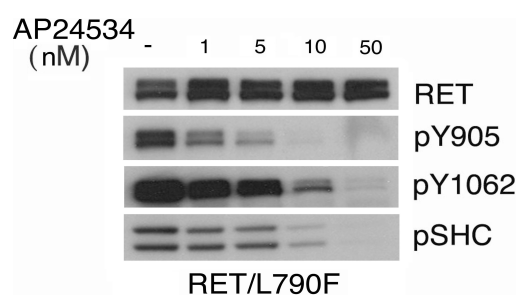


Fig. 19: Ponatinib inhibits in vivo phosphorylation of RET mutant in L790 position. For a description, see legend to Figure 15

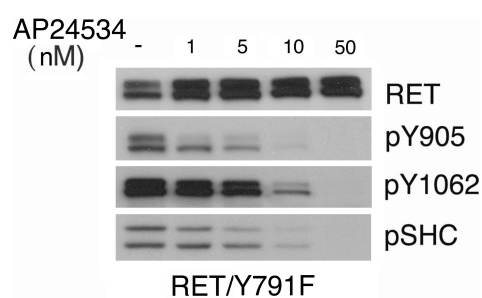


Fig. 20: Ponatinib inhibits in vivo phosphorylation of RET mutant in Y791 position. For a description, see legend to Figure 15

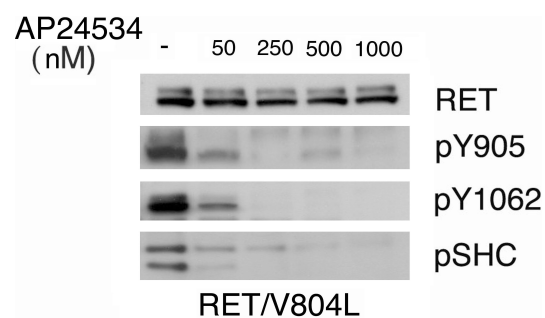


Fig. 21: Ponatinib inhibits in vivo phosphorylation of RET mutant in V804 position (V804L). For a description, see legend to Figure 15

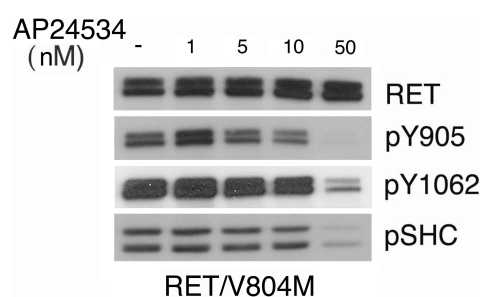


Fig. 22: Ponatinib inhibits in vivo phosphorylation of RET mutant in V804 position (V804M). For a description, see legend to Figure 15

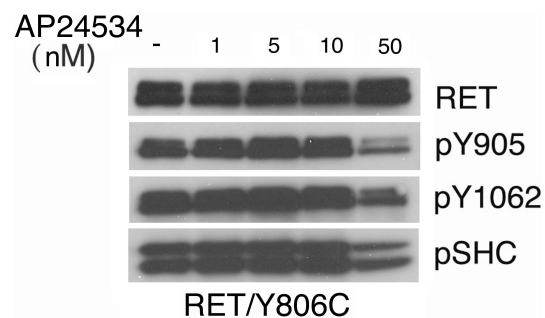


Fig. 23: Ponatinib inhibits in vivo phosphorylation of RET mutant in Y806 position. For a description, see legend to Figure 15

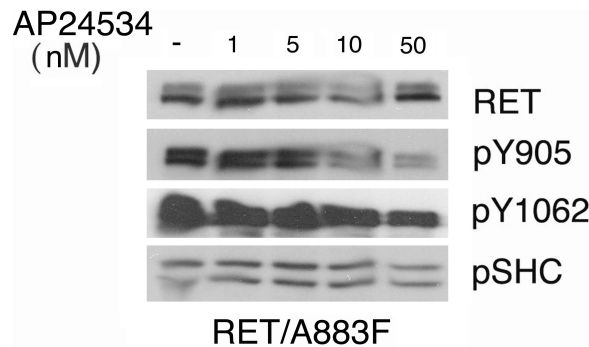


Fig. 24: Ponatinib inhibits in vivo phosphorylation of RET mutant in A883 position. For a description, see legend to Figure 15

4.1.3 Ponatinib efficiently inhibits RET-mediated cancer cell proliferation.

Then, we asked whether ponatinib-mediated RET kinase inhibition was translated into cell proliferation blockade. To this aim, growth curves were performed by using different doses of compound and NIH3T3 transfectants expressing RET/C634R or RET/M918T mutants. As control, we used HRAS/G12V-transformed or untransfected NIH3T3 cells. Growth curves reported in **Figures 25-28** demonstrate that ponatinib blunted cell proliferation at nM concentration and that this effect was RET-dependent, as it was more intense in cells expressing RET than RET-negative cells.

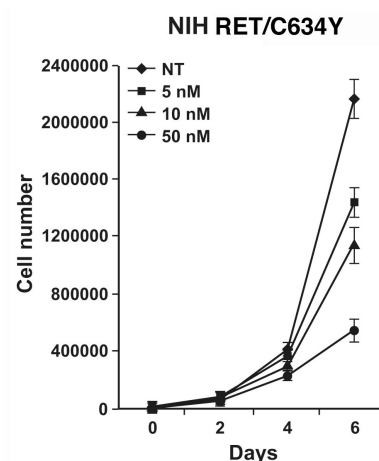


Fig. 25: Ponatinib inhibits proliferation of NIH3T3 cells transformed by RET/C634R. Cells were plated in triplicate, treated with indicated doses of ponatinib (NT: not treated) and counted at different time points. Average results of 3 independent determinations are reported \pm S.D.

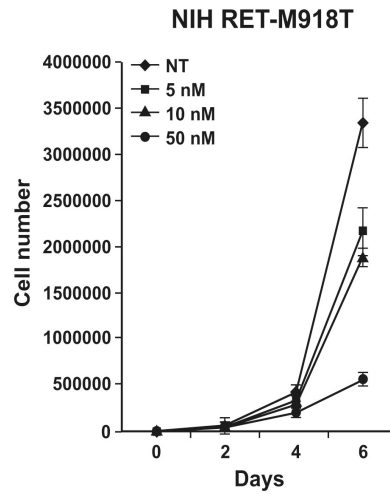


Fig. 26: Ponatinib inhibits proliferation of NIH3T3 cells transformed by RET/M918T. Cells were plated in triplicate, treated with indicated doses of ponatinib (NT: not treated) and counted at different time points. Average results of 3 independent determinations are reported \pm S.D.

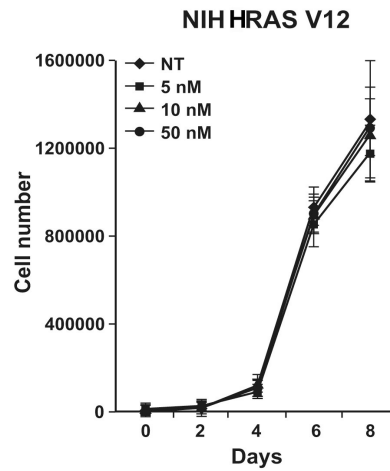


Fig. 27: Ponatinib exerts only mild proliferative inhibition of NIH3T3 cells transformed by HRAS. Cells were plated in triplicate, treated with indicated doses of ponatinib (NT: not treated) and counted at different time points. Average results of 3 independent determinations are reported \pm S.D.

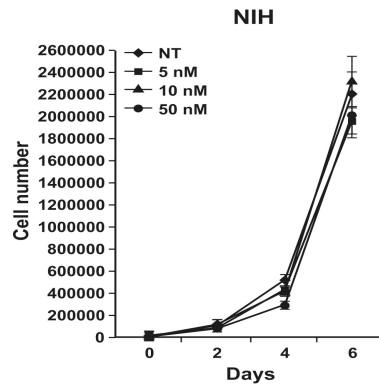


Fig. 28: Ponatinib exerts only mild proliferative inhibition of wild type NIH3T3 cells. Cells were plated in triplicate, treated with indicated doses of ponatinib (NT: not treated) and counted at different time points. Average results of 3 independent determinations are reported \pm S.D.

Finally, we measured proliferative effects of ponatinib on thyroid cancer cells endogenously expressing RET mutants. We selected TT cells (MTC harbouring C634W RET mutation) and TPC1 cells (PTC cells harbouring RET/PTC1 chimeric oncogene); as control, we used BCPAP (PTC cell line harbouring BRAF V600E mutation and no RET mutation) and Nthy-ori 3-1, normal thyrocytes immortalized by Large T SV40. **Figures 29-32** show that ponatinib had potent anti-proliferative effects in RET-positive cancer cells. In TT cells, effects were seen at doses (1-5 nM) even smaller than IC₅₀ for RET kinase (10 nM), suggesting that targets other than RET can contribute to such a potent growth inhibitory activity or that MTC cells are highly susceptible even to minor reduction of RET kinase activity.

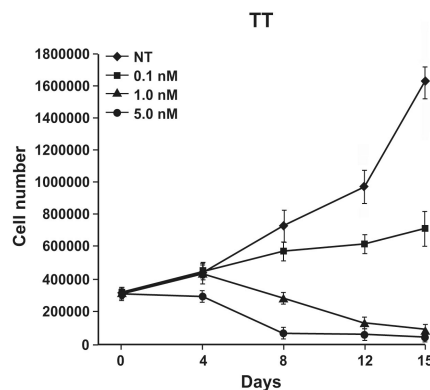


Fig. 29: Ponatinib inhibits proliferation of RET/C634W-positive TT cells. Cells were plated in triplicate, treated with indicated doses of ponatinib (NT: not treated) and counted at different time points. Average results of 3 independent determinations are reported \pm S.D.

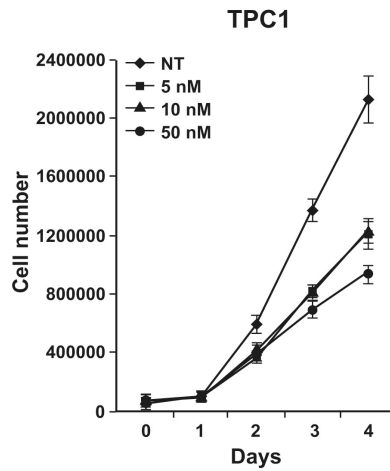


Fig. 30: Ponatinib inhibits proliferation of RET/PTC1-positive TPC1 cells. Cells were plated in triplicate, treated with indicated doses of ponatinib (NT: not treated) and counted at different time points. Average results of 3 independent determinations are reported \pm S.D.

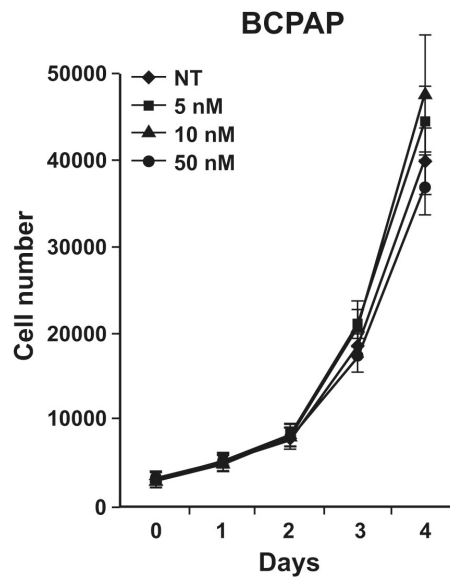


Fig. 31: Ponatinib exerts only mild proliferative inhibition of BRAF/V600E (RET wild type) BCPAP cells. Cells were plated in triplicate, treated with indicated doses of ponatinib (NT: not treated) and counted at different time points. Average results of 3 independent determinations are reported \pm S.D.

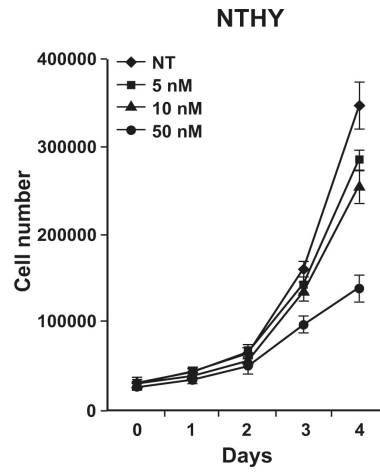


Fig. 32: Ponatinib exerts only mild proliferative inhibition of SV40-LT-immortalized (RET wild type) normal thyrocytes. Cells were plated in triplicate, treated with indicated doses of ponatinib (NT: not treated) and counted at different time points. Average results of 3 independent determinations are reported \pm S.D.

4.1.4 Ponatinib efficiently inhibits RET-mediated tumor growth in mouse xenografts.

Finally, we sought to determine whether ponatinib had inhibitory effects *in vivo* against RET-dependent tumor growth. To this aim, we injected nude mice with 5×10^7 TT cells. After approximately 30 days, when tumors measured ~ 70 - 100 mm^3 , animals (10 for each group) were randomly assigned to receive *per os* ponatinib (30 mg/kg/day) or vehicle 5 days/week for three weeks. Tumor size was monitored with calipers initially every 7 days and then every 3 days. Treatment with ponatinib strongly reduced tumor growth (**Figure 33**). Mice were sacrificed after 3 weeks, when tumors in vehicle treated mice were very large while in most ponatinib-treated mice they were virtually undetectable. Importantly, we noted no sign of wasting in treated mice.

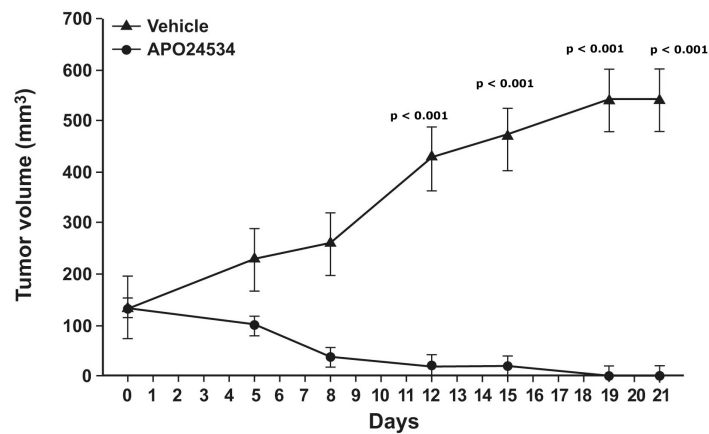


Fig. 33: Ponatinib exerts potent anti-tumorigenic activity in nu/nu mice xenografted with TT cells.

4.2 ISOLATION OF PLX4032 RESISTANT THYROID CANCER CELLS AND CHARACTERIZATION OF THE MECHANISMS OF RESISTANCE.

4.2.1 Chronic BRAF Inhibition leads to Acquired Drug Resistance

We have previously reported that vemurafenib (PLX4032) was able to reduce proliferation of BRAF mutant thyroid cancer cells in culture (Salerno et al. 2010). To investigate if chronic BRAF inhibition could lead to drug resistance, a panel of thyroid cancer cell lines harboring the V600E mutation in the BRAF gene [BCPAP (V600E/V600E) (PTC), BHT101 (WT/V600E) (ATC) and SW1736 (WT/V600E) (ATC)], were chronically treated with increasing concentrations of PLX4032 (**Figure 34**). This was done following two different protocols:

- (i) Parental cells were treated with step-by-step increased PLX4032 concentration starting from 100 nM up to 2 μ M (method R1);
- (ii) Parental cells were initially treated with high dose (5 μ M) of PLX4032 for 12 hrs, and then subjected to scaling-up, starting from 100nM to reach 2 μ M in a step-wise manner (method R2).

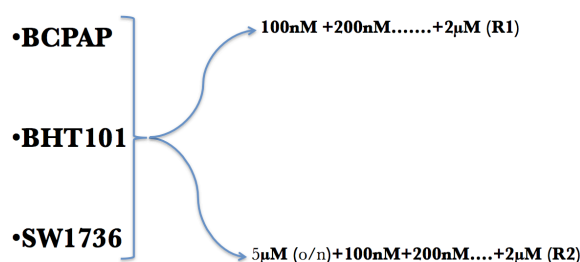


Fig. 34: Schematic representation of generation of PLX4032 resistant cells.

Both methods, successfully allowed us to get resistant sub-lines (R1 & R2) able to grow at 2 μ M of PLX4032. Since, the PLX4032 resistant cells R1 and R2 did not behave differently, we selected BCPAP-R1, BHT101-R1 and SW1736-R1 cells for further experiments.

Treatment of parental cells (BCPAP-P, BHT101-P & SW1736-P) with PLX4032 reduced viability with Inhibitory Concentrations 50 (IC₅₀) of about 500 nM (IC₅₀; BCPAP-P = 444nM, BHT101-P = 240nM & SW1736-P = 400nM). Noteworthy, significantly higher doses of PLX4032 were required to achieve 50% growth inhibition in resistant cells (IC₅₀; BCPAP-R = >5 μ M, BHT101-R = 2.9 μ M & SW1736-R = > 5 μ M), (**Figure 35**). These data demonstrate that chronic treatment with a specific BRAF inhibitor can lead to development of resistance in thyroid carcinoma harboring BRAF V600E mutation.

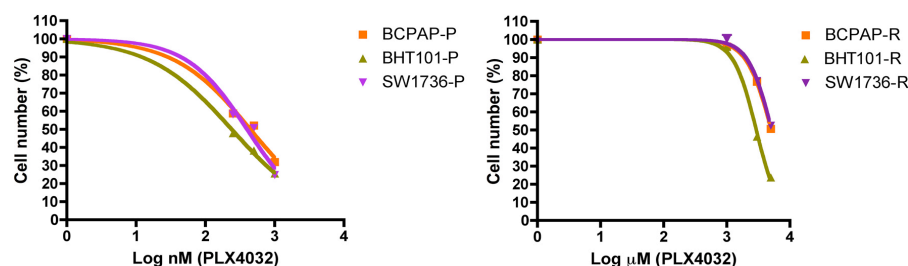


Fig. 35: BRAF mutant cell lines chronically treated with PLX4032 develop drug resistance. Sensitivity of parental (IC₅₀ 200 – 450nM) and corresponding resistant cells (IC₅₀ > 3-5 μM) to PLX4032 by proliferation assay. Cells were exposed to different doses of compound for 72 hr and then counted in triplicate: data are reported as % of cell number. Sensitivity of parental and resistant cells was significantly different ($p < 0.001$) at dose 1 μM.

All parental and resistant cells were genotyped by sequencing the 15th exon of BRAF using Sanger's method. The sequencing result confirmed the presence of BRAF V600E mutation in parental cells and that this mutation was kept in resistant cells, as well (**Figure 36**).

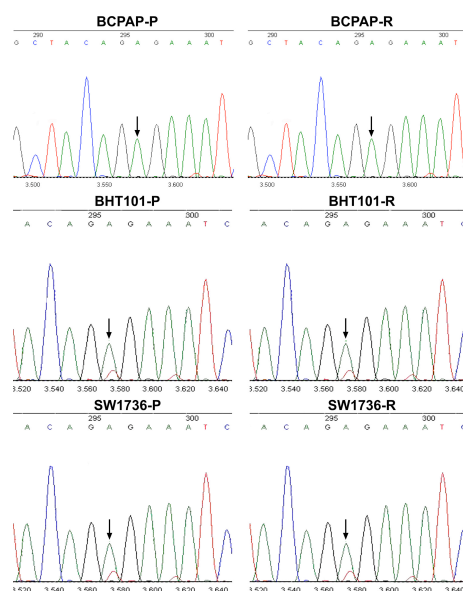


Fig. 36: BRAF V600E mutation in parental and PLX4032-resistant thyroid carcinoma cell lines.

Altogether, these findings demonstrate that thyroid cancer cells can develop resistance to PLX4032 while keeping the BRAF V600E mutation.

4.2.2 PLX4032-resistant cells feature increased migration and motility

To characterize the phenotype of PLX4032-resistant thyroid carcinoma cells, we investigated proliferation, invasion through Matrigel and motility. No significant differences were noted in the growth rate between parental and resistant cells (data not shown). In contrast, resistant cells featured increased invasion through Matrigel (**Figure 37**) and increased motility on the plastic dish (**Figure 38**). Moreover, while parental cell motility and invasion were sensitive to PLX4032 treatment, as expected, resistant cells were refractory to the treatment.

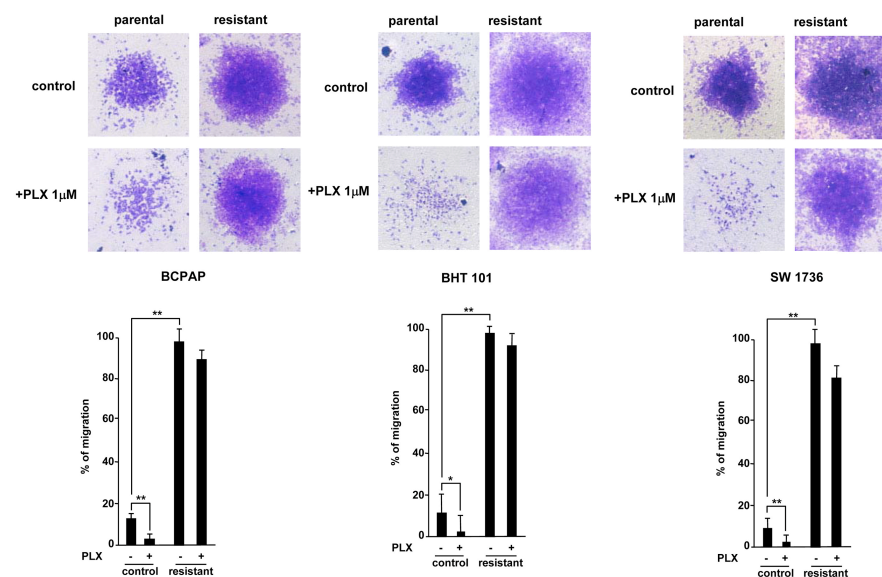


Fig. 37: PLX4032-resistant cells display increased invasion through Matrigel. Cells were seeded in upper chamber of transwells coated with Matrigel and incubated for 12 h in the presence or not of PLX4032 (1 μ M); the upper surface of the filter was wiped clean, and cells on the lower surface were stained and counted. Invasive ability was expressed as number of invaded cells. Values represent the average of triplicate experiments \pm SD. **, $P < 0.001$. Note that resistant cells display increased invasion with respect to parental cells and that such invasion, differently from parental cells, does not respond to PLX4032 treatment.

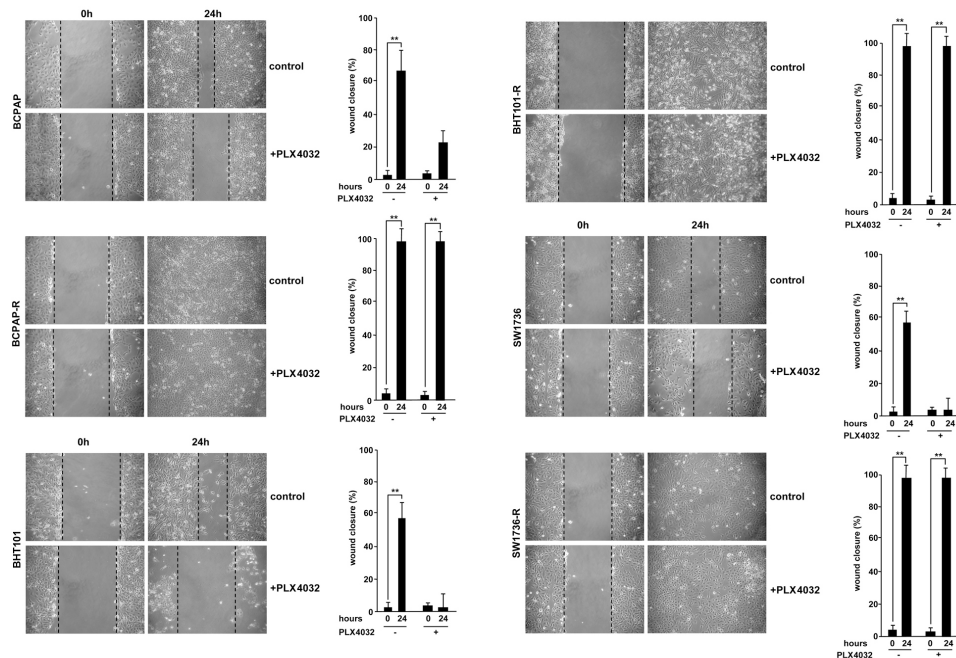


Fig. 38: PLX4032-resistant cells display increased motility. Wound healing assay: cells were plated at confluence and scratch wounds were inflicted. After 24h (with cells treated or not with PLX4032 1μM), cell plates were photographed and cells closing the wound were counted. Wound closure was measured by calculating pixel densities in the wound area and expressed as percentage of wound closure of triplicate areas \pm SD. The average results of three experiments are reported with bars representing 95% confidence intervals; (**, $p < 0.001$). Note that resistant cells display increased motility with respect to parental cells and that such motility, differently from parental cells, does not respond to PLX4032 treatment.

Altogether, these findings demonstrate that thyroid cancer cells resistant to PLX4032 acquired a more malignant phenotype, that is characterized by increased motility and invasion.

4.2.3 PLX4032-resistant cells feature altered intracellular signaling

To investigate the mechanisms of PLX4032-resistance in vitro, we used phosphospecific antibodies to probe the activation status of the RAF downstream effectors, MEK1/2 and ERK1/2, in parental versus resistant cells, with and without PLX4032 (**Figure 39**).

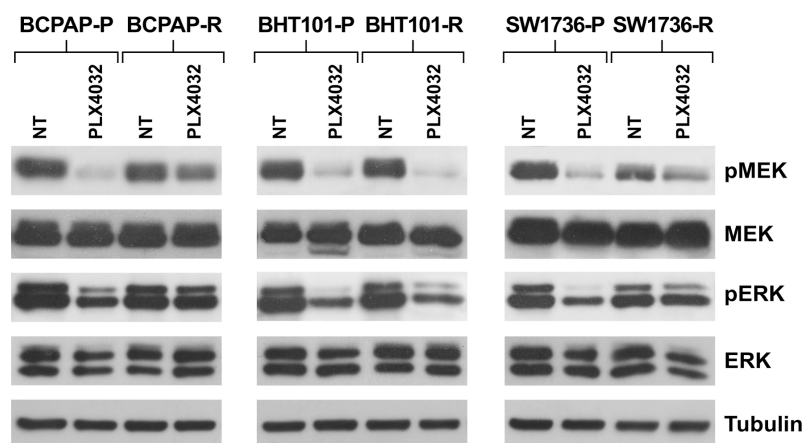


Fig. 39: PLX4032-resistant cells display differential ERK activation. Parental and PLX4032-resistant cells were treated with PLX4032 (1 μ M) and the effects on ERK signaling were determined by immunoblotting for pMEK1/2 and pERK1/2 levels. Total MEK1/2, ERK1/2 and tubulin levels are reported as loading controls.

As expected, PLX4032 decreased pMEK1/2 and pERK1/2 in all parental cells (P). As far as resistant cells (R), the pattern of MEK-ERK phosphorylation varied among resistant cells, suggesting distinct mechanisms. BCPAP-R showed strong resistance to PLX4032-induced MEK/ERK inhibition (suggesting MAPK pathway reactivation). Instead, BHT101-R (totally) and SW1736-R (partially) remained sensitive to PLX4032-mediated decrease in the levels of p-MEK1/2 and p-ERK1/2 (suggesting bypass through an alternative signaling pathway).

To search for the mechanism of pMEK resistance in BCPAP-R, we observed that PLX4032-resistant cells featured increased levels of phosphorylation of CRAF at 338 site, a marker of CRAF activation (**Figure 40**). This was not observed in BHT101 and SW1736 resistant cells (data not shown). CRAF activation in BCPAP-R could be a suitable explanation of pMEK/pERK resistance to PLX4032 treatment and of to the growth, invasion and motility inhibitory effects of the drug (see also below). The molecular mechanism for CRAF phosphorylation remains unclear. By Sanger sequencing we excluded mutations in RAS that can favour the formation of

BRAF/CRAF heterodimers; we also excluded secondary mutations in BRAF that can impair drug binding (data not shown)

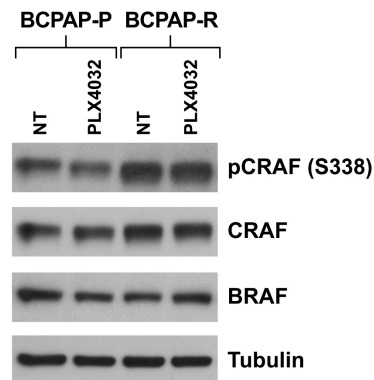


Fig. 40: PLX4032-resistant BCPAP-R feature CRAF phosphorylation. Parental and PLX4032-resistant cells were treated with PLX4032 (1 μ M) and the effects on BRAF and CRAF signaling were determined by immunoblotting for pCRAF (S338) phosphorylation levels. Total CRAF, BRAF and tubulin levels: loading controls.

Whatever the mechanism, we checked whether MEK inhibitors could overcome PLX4032 resistance; this was particularly important for BCPAP-R cells in which we showed that resistance to PLX4032 was accompanied by inability of PLX4032 of reducing pMEK. As a control, we initially showed that U0126 was able to block pERK as expected (**Figure 41**).

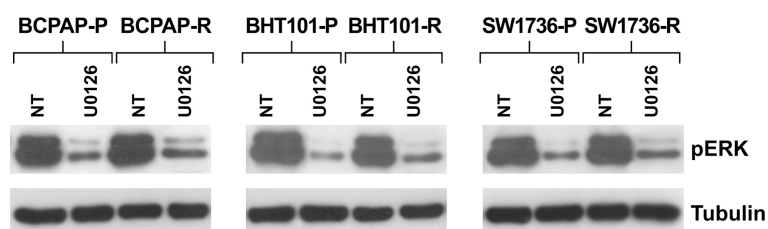


Fig. 41: Effects of MEK inhibitor (U0126). Immunoblots showing pERK levels in parental and resistant cells treated with DMSO (NT) or 10 μ M U0126 for 1 h.

Thereafter, we checked cell growth inhibition by U0126. Noteworthy, the three resistant cell lines (including BCPAP-R) were quite resistant not only to PLX4032 but also to U0126 (IC₅₀ increased from 3.9 to almost 6.2 μ M), consistent with a model whereby these resistant cells have switched from

BRAF-ERK to a compensatory pathway for proliferation (**Figure 42**). This applied also to BCPAP-R cells; therefore also for these cells resistance cannot be explained by the observed pMEK rescue through CRAF.

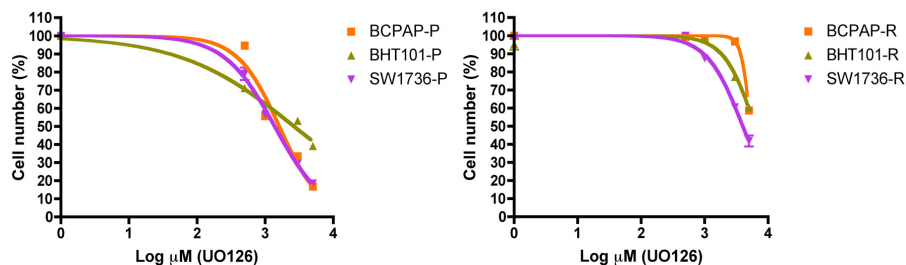


Fig. 42: Effects of MEK inhibitor (UO126). Proliferation curves for parental and resistant cells treated with the indicated UO126 for 96 h (relative to DMSO-treated controls).

4.2.4 AKT pathway activation in PLX4032-resistant cells.

By searching for alternative pathway activation that may mediate resistance, we found that all the three resistant cells featured up regulation of pAKT (Ser473) (site of phosphorylation by TORC2/PDK2 complex) (**Figure 43**).

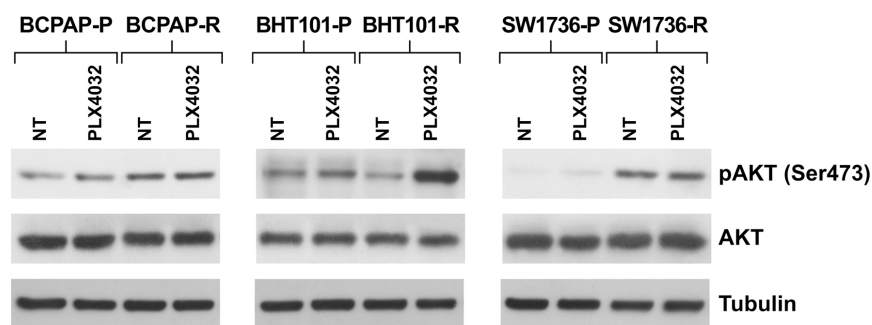


Fig. 43: AKT pathway upregulation in PLX4032-resistant cells. Cells were treated with PLX4032 (1μM) and the effects on AKT signaling were determined by immunoblotting for pAKT (Ser473) levels. Total AKT and tubulin levels: loading controls.

There were however differences among the resistant cells, with BCPAP-R and SW1736-R featuring constitutive potentiation of pAKT (Ser473). Instead, BHT-101-R featured only a modest baseline potentiation of pAKT (Ser473), but AKT phospho-levels strongly increased upon PLX4032 treatment.

Moreover, in BCPAP-R and SW1736-R resistant cells, increased AKT phosphorylation also observed at the residue Thr308 (PDK1 site), which indicates activation of some upstream signaling event (as examples: RTK or PI3K activation, PTEN loss). We did not observe the same feature in BHT101-R (**Figure 44**).

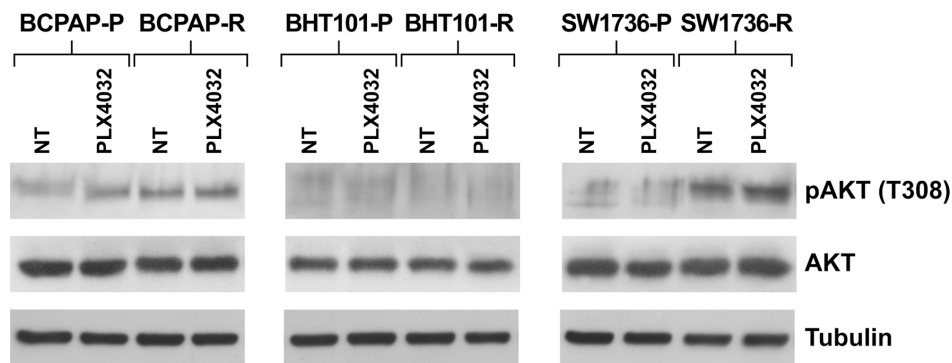


Fig. 44: AKT pathway upregulation in PLX4032-resistant cells. Cells were treated with PLX4032 (1 μ M) and the effects on AKT signaling were determined by immunoblotting for pAKT (T308). Total AKT and tubulin levels: loading controls.

Thus, we checked sensitivity of PLX4032-resistant cells to PI103, a double PI3K α and mTOR inhibitor to study the relevance of the PI3K/AKT/mTOR cascade in mediating resistance. Growth of both parental and resistant cells was equally inhibited (**Figure 45**). This indicates that inhibition of AKT pathway can overcome the PLX4032 resistance.

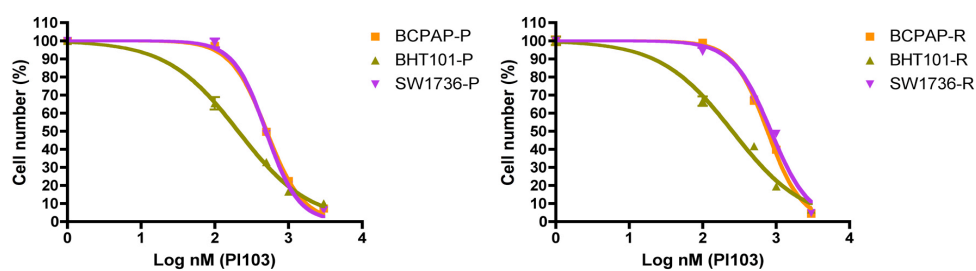


Fig. 45: Compensatory signaling inhibition limits the growth of PLX4032 resistant cells. Proliferation curves of parental and resistant cells treated with the indicated PI103 concentrations for 96 h (relative to DMSO-treated controls).

One possibility to explain AKT pathway activation was downregulation of the PTEN phosphatase, a negative regulator of AKT phosphorylation. However, PTEN altered expression was excluded by Q-RT-PCR (data not shown).

5. DISCUSSION

This dissertation is focused on the study of molecular targeted therapeutic approaches for thyroid cancer and, in particular, on mechanisms of resistance to molecular targeting of BRAF and RET kinases.

Most well-differentiated thyroid carcinomas (PTC and FTC) are effectively treated by surgery, adjuvant radio-iodine ablation and TSH suppression. In contrast, there is a urgent need of novel therapeutic measures for thyroid carcinomas that have lost radio-iodine concentration ability (PDTC and ATC) as well as for MTC, a neuroendocrine cancer deriving from C-cells that do not express sodium-iodine symporter (NIS) (Cooper et al. 2009; Schlumberger et al. 2008).

PTC often features oncogenic activation of BRAF serine/threonine kinase and MTC is associated to oncogenic conversion of RET tyrosine kinase (Nikiforov et al. 2011). BRAF mutation (most commonly V600E) is found in about half PTC and one third of ATC. Though still quite controversial, correlative studies in human patients have suggested that PTC with aggressive features (including large tumor size, distant metastases, development of radio-iodine refractory disease, recurrence) are commonly associated to BRAF mutation (Xing 2007). Importantly, experimental models have supported this concept by demonstrating that BRAF is able to stimulate the expression of genes involved in invasion and metastatic dissemination and to obstruct markers of thyroid differentiation (Nucera et al. 2010; Mesa et al. 2006). One mechanism by which BRAF-driven constitutive MAPK pathway activation inhibits NIS expression is based on a TGF- β autocrine loop, which downregulates Pax8 and evokes a Smad3-dependent inhibition of Pax8 binding to the NIS promoter (Riesco-Eizaguirre et al. 2009). Consistently, transgenic mice featuring thyroid targeted expression of BRAF V600E were shown to develop PTC rapidly evolving to poorly-differentiated carcinomas and BRAF pathway blockade was demonstrated to restore thyroid differentiation and radio-iodine uptake in this mouse model (Knauf et al. 2005; Chakravarty et al. 2011).

MTC is associated to RET mutation in about half of sporadic cases and virtually all familial cases and RET mutation, particularly M918T was demonstrated to correlate with an aggressive clinical behaviour (Elisei et al. 2008).

Oncogenic kinases like RET and BRAF can be targeted with small molecule organic compounds (PKI) that by binding the ATP pocket (Type 1 and Type 2 inhibitors) can obstruct kinase function and induce cancer cell death or proliferative arrest (Sherman 2011). Several RET and BRAF PKIs have been identified and their efficacy studied in preclinical models and in some cases in clinical trials. The most advanced RET PKIs, are vandetanib and cabozantinib (XL184); these compounds target RET as well as other receptors including VEGFR (in the case of both) and EGFR (in the case of

vandetanib) and therefore are able to exert their effects on several targets in tumor cells and tumor stroma (Wells et al. 2011; Kurzrock et al. 2011). One BRAF PKI, vemurafenib (PLX4032), has been demonstrated to exert activity in preclinical models of thyroid cancer (Salerno et al. 2010; Xing et al. 2011; Nucera et al. 2009; Nucera et al. 2010; Nehs et al. 2010). Vemurafenib recently elicited spectacular clinical regressions of the disease in the large majority of metastatic melanoma patients, which were positive for BRAF mutation (Bollag et al. 2010; Chapman et al. 2011).

It is well-established that cancers can escape treatment with PKIs by acquiring resistance and two paradigms of molecular resistance formation have been depicted. In one case, cancer cells develop secondary mutations in the protein kinase that is targeted by the drug, impairing drug binding. In the second case, cancer cells feature activation of compensatory signaling pathway that bypass the drug-elicited block.

The first model is illustrated by the development of resistance to imatinib (Gleevec) in CML patients secondary to the development of point mutations in BCR-ABL that, either directly block drug binding or allosterically change the conformation of the kinase to which imatinib binds. Among these mutations, T315I mutation affecting the so-called gate-keeper residue is the most commonly found and revealed to be the most difficult to deal with. Importantly, mutations in corresponding residues have been found to mediate resistance of oncogenic kinases other than BCR-ABL to their corresponding PKIs, including T790M for EGFR, T670I for KIT, T674I for PDGFR α and T681I for PDGFR β (Druker 2006). RET mutations in the gate-keeper residue (V804M, V804L) have been demonstrated to cause resistance to vandetanib and though these mutations have not been yet described as secondary mutations developed upon treatment with the drug, they are pathogenetic mutations causing MTC and they are particularly prevalent in some geographic areas including Italy (Pinna et al. 2007; Romei et al. 2010). Thus far, mutations in BRAF have not been described to cause resistance to BRAF PKIs (Poulikakos et al. 2011).

The second model is illustrated by resistance formation to EGFR family PKIs that involved the up-regulation of alternative receptors, like MET and PDGFR, ultimately leading to rescue of cancer cell growth (Sergina et al. 2007; Stommel et al. 2007). Often such a switch involved activation of the PI3K/AKT signaling cascade, a pathway that is commonly involved in cancer cell treatment resistance (Villanueva et al. 2010; Paraiso et al. 2011). Recently, molecular mechanisms of resistance to BRAF PKI in melanoma cells have been identified and they fit with this second model. Indeed, melanoma cells resistant to BRAF blockade either developed activation of NRAS and COT kinase, or activation of PDGFR. Activation of NRAS or COT bypassed BRAF block sustaining MAPK signaling via CRAF or MEK, respectively. Instead, PDGFR activation likely sustained cell proliferation by stimulating pathways alternative to the MAPK one (Solit et al. 2010; Nazarian et al. 2010; Johannessen et al. 2010; Poulikakos et al. 2011).

In this framework, this Dissertation has been focused on RET and BRAF PKI with a view of exploring mechanisms of resistance and possible strategies to overcome it. Specifically, since it is known that some RET mutations confer resistance to PKIs, in a first part of the Dissertation we have looked at compounds able to function against these RET mutants; in the second part of the work, since no information is currently available about resistance of thyroid cancer cells to BRAF PKIs, we have isolated and studied cells resistant to vemurafenib.

As far as RET, here we provide evidence that ponatinib (AP24534) is a novel RET PKI. Ponatinib is a multitargeted Type 2 kinase inhibitor that inhibits a set of kinases, including BCR-ABL, FLT3, KIT, FGFR1, and PDGFR α at clinically achievable drug levels (Gozgit et al. 2011; Zhou et al. 2011; Huang et al. 2010; O'Hare et al. 2009). Prompted by the observation that ponatinib inhibits also T315I BCR-ABL (O'Hare et al. 2009), we explored activity of this compound against RET and RET (V804 and Y806) drug-resistant mutants. We show that ponatinib is a potent RET PKI, with nanomolar activity in RET phosphorylation assays (IC₅₀: 10-50 nM). Ponatinib inhibited RET-driven proliferation of fibroblasts transformed by RET derived oncogenes and also the proliferation of thyroid carcinoma cells (both PTC and MTC) bearing RET-derived (RET/PTC and RET/MEN2) oncogenes. Finally, ponatinib significantly reduced growth of MTC cells (TT) tumor xenografts in nude mice. However, it is possible that inhibition of multiple targets besides RET can concur to these in vitro and in vivo anti-neoplastic effects. This is supported by the observation that inhibition of MTC cell proliferation in vitro was noted at doses even smaller than IC₅₀ for the RET kinase, suggesting that the drug was probably acting on multiple targets. Importantly, ponatinib was effective also against V804 and Y806 RET mutants at doses only slightly higher than those required to inhibit the other RET mutants tested. This raises the possibility that this drug may be useful for MTC patients bearing such RET mutations. We and others have previously reported that sorafenib was active against RET V804 (Carlomagno et al. 2006; Plaza-Menacho et al. 2007); however, this drug did not achieve very potent results in a small Phase II study in MTC patients (Lam et al. 2010). Being ponatinib in advanced clinical experimentation in patients affected by leukemia, overall, our data suggest that this drug may be worth testing also in patients affected by MTC who failed previous treatments.

As far as BRAF targeting, PLX4032 (vemurafenib) was previously reported to have cytostatic and anti-tumorigenic activity against thyroid cancer cells, but no mechanism of resistance was previously known. Thus, we started by selecting BRAF mutant thyroid cancer cells (both PTC- and ATC-derived) able to proliferate also in the presence of PLX4032 (2 μ M concentration). According to what has been observed in melanoma, PLX4032-resistant thyroid cancer cells did not feature secondary BRAF mutation in the gate-keeper residue (data not shown), thus pointing to pathway switch as a likely mechanism of resistance. Two out of the three PLX4032-resistant cells were

still susceptible to PLX4032-mediated inhibition of MAPK (MEK and ERK) phosphorylation, thus suggesting that resistance is not mediated by MAPK rescue but rather to compensatory activation of another(s) pathway. Instead, PLX4032-resistant BCPAP cells featured sustained pMEK and pERK levels even when treated with high doses of drug. Mechanistically, this can be explained by compensatory activation of CRAF, since we noted increased activatory CRAF phosphorylation in PLX4032-resistant BCPAP cells. Whatever the mechanism, it is unlikely that stimulation of MAPK pathway is an important component of resistance to PLX4032 because all the three PLX4032-resistant cell lines featured also resistance to MEK inhibition. Thus, it can be envisaged that activation of non-MAPK based signaling pathways may cause such resistance. Accordingly, PLX4032-resistant cells featured increased aggressiveness with respect to parental cells characterized by high levels of Matrigel invasion and cell motility, a phenotype that is in agreement with the possibility that these cells have developed activation of signaling pathways in addition to the MAPK one. We found that 2 out of the 3 PLX4032-resistant cells featured increased phosphorylation of AKT. Moreover, preliminary data suggest that also AKT downstream effectors such as pS6 have an increased phosphorylation in PLX4032-resistant cells. The mechanism of AKT stimulation is still unknown. We excluded RAS mutations and PTEN downregulation as well as increased AKT expression (data not shown); AKT and PIK3CA sequencing are in progress to verify whether mutations at these levels may contribute to pathway activation. Furthermore, preliminary data suggest that RTK phosphorylation, in particular IGF1R, may be involved. Whatever the mechanism, our data demonstrate that, PLX4032-resistant cells were sensitive to a PI3K-mTOR double inhibitor, thereby suggesting the use of these inhibitors alone or in combination with PLX4032 as possible strategy to circumvent resistance.

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Attached Manuscript #I

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The β -Catenin Axis Integrates Multiple Signals Downstream from RET/Papillary Thyroid Carcinoma Leading to Cell Proliferation

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Abstract

RET/papillary thyroid carcinoma (RET/PTC) oncoproteins result from the in-frame fusion of the RET receptor tyrosine kinase domain with protein dimerization motifs encoded by heterologous genes. Here, we show that RET/PTC stimulates the β -catenin pathway. By stimulating PI3K/AKT and Ras/extracellular signal-regulated kinase (ERK), RET/PTC promotes glycogen synthase kinase 3 β (GSK3 β) phosphorylation, thereby reducing GSK3 β -mediated NH₂-terminal β -catenin (Ser33/Ser37/Thr41) phosphorylation. In addition, RET/PTC physically interacts with β -catenin and increases its phosphotyrosine content. The increased free pool of S/T(nonphospho)/Y(phospho) β -catenin is stabilized as a result of the reduced binding affinity for the Axin/GSK3 β complex and activates the transcription factor T-cell factor/lymphoid enhancer factor. Moreover, through the ERK pathway, RET/PTC stimulates cyclic AMP-responsive element binding protein (CREB) phosphorylation and promotes the formation of a β -catenin-CREB-CREB-binding protein/p300 transcriptional complex. Transcriptional complexes containing β -catenin are recruited to the cyclin D1 promoter and a cyclin D1 gene promoter reporter is active in RET/PTC-expressing cells. Silencing of β -catenin by small interfering RNA inhibits proliferation of RET/PTC-transformed PC Cl3 thyrocytes, whereas a constitutively active form of β -catenin stimulates autonomous proliferation of thyroid cells. Thus, multiple signaling events downstream from RET/PTC converge on β -catenin to stimulate cell proliferation. [Cancer Res 2009;69(5):1867–76]

Introduction

Papillary thyroid carcinoma (PTC) features chromosomal aberrations that result in the fusion of the tyrosine kinase domain of the RET receptor with the NH₂ terminus of heterologous proteins, thereby generating the RET/PTC oncoproteins (1). Although there is large variation according to the geographic area and the detection method, the fraction of PTC samples positive for RET/PTC oncogenes is estimated to be 20% to 40%; this fraction increases up to 80% in PTC developed in radiation-exposed individuals (1). RET/PTC1 (H4-RET) and RET/PTC3 (NCOA4-RET) are the most prevalent variants (1). Fusion with protein partners that have protein-protein interaction motifs provides RET/PTC

kinases with dimerizing interfaces, thereby resulting in ligand-independent dimerization and constitutive kinase activation. Autophosphorylation of RET Y1062 plays a particularly important role in cell transformation (2). Accordingly, when phosphorylated, Y1062 acts as the binding site for several protein tyrosine binding proteins, namely, Shc, IRS1/2, FRS2, and DOK1/4/5 (2). This mediates recruitment of growth factor receptor binding protein 2/son of sevenless homologue complexes (2–5) and Gab-family adaptors (2, 6–8) leading to Ras/RAF/extracellular signal-regulated kinase (ERK) and PI3K/AKT signaling (9–11). Moreover, RET/PTC depends on the phosphorylation of Y1062 for recruitment at the inner surface of the cell membrane (2–5).

RET/PTC-mediated transformation requires the Ras/RAF/ERK cascade (11–13). In line with this observation, oncogenic conversion of a member of the RAF family, BRAF, is frequently detected in PTC (1, 14). In addition, deregulation of the PI3K signaling, through activation of PI3K and AKT serine/threonine kinase or loss of PTEN phosphatase, is prevalent in thyroid cancer (15, 16). PI3K signaling is mitogenic for thyrocytes (15, 16).

β -Catenin is a multifunctional protein that plays an important role in signal transduction. In normal resting cells, β -catenin is mainly localized to the adherens junctions, whereas free cytosolic β -catenin is recruited to a "destruction" complex that includes the scaffolding protein Axin, the tumor suppressor adenomatous polyposis coli (APC), and glycogen synthase kinase 3 β (GSK3 β). This complex facilitates the NH₂-terminal serine/threonine phosphorylation of β -catenin by GSK3 β , thereby targeting β -catenin for degradation by the ubiquitin-proteasome (17). When the pathway is activated, as it occurs in the presence of Wnt ligands, β -catenin is stabilized. Stabilization of β -catenin results in its nuclear accumulation and interaction with T-cell factor/lymphoid enhancer factor (TCF/LEF) or other transcription factors and in activation of genes required for cell proliferation (e.g., *c-Myc* and *cyclin D1*; ref. 18). PI3K/AKT induces Ser9/21 GSK3 β phosphorylation and inhibition of GSK3 β -mediated phosphorylation of β -catenin (19). p90RSK, a Ras/ERK downstream kinase, also phosphorylates and inhibits GSK3 β thereby leading to β -catenin up-regulation (17, 20, 21). In this pathway, ERK associates with a docking motif, FKBP (residues 291–294), of GSK3 β and phosphorylates it at Thr43 to prime it for subsequent phosphorylation at Ser9/21 by p90RSK (22).

Up-regulation of β -catenin occurs in a variety of cancers, namely, colorectal, breast, and ovarian cancers (17, 18). Mutations that activate β -catenin occur late in thyroid tumor progression, being detected in undifferentiated (anaplastic) thyroid carcinomas (23). Increased free β -catenin pools have been observed in thyroid carcinomas secondary to reduced E-cadherin expression (24). It has been recently reported that oncogenic point mutants of RET (2A-RET and 2B-RET), which are associated with medullary thyroid

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cancer, phosphorylate β -catenin on Y654 thereby promoting β -catenin escape from APC/Axin/GSK3 β -mediated destruction (25). This prompted us to investigate the functional connection between RET/PTC and the β -catenin signaling cascades.

Materials and Methods

Cell lines. HEK293T cells were grown in DMEM supplemented with 10% FCS (Invitrogen). The human thyroid cancer cell lines, derived from papillary (TPC-1 and BCPAP) or anaplastic (OCUT-1 and 8505C) thyroid carcinoma, were grown in DMEM containing 10% fetal bovine serum (FBS). 8505C were from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). OCUT-1 was a gift of K. Hirakawa and N. Onoda (Osaka, Japan). TPC-1 harbors a RET/PTC1 rearrangement; BCPAP, OCUT-1, and 8505C harbor a BRAF V600E mutation (26). Normal human thyrocytes were isolated from normal thyroid tissue obtained from a patient who underwent thyroid surgery and cultivated in RPMI supplemented with 20% FBS. PC Cl3 (hereafter called "PC") is a differentiated thyroid follicular cell line derived from 18-mo-old Fischer rats. PC cells were cultured in Coon's modified Ham's F-12 medium supplemented with 5% calf serum and a mixture of six hormones, including thyrotropin (10 milliunits/mL), hydrocortisone (10 nmol/L), insulin (10 μ g/mL), apo-transferrin (5 μ g/mL), somatostatin (10 ng/mL), and glycyl-histidyl-lysine (10 ng/mL; Sigma). RET/PTC-expressing PC cells have been described previously (11). To obtain PC cells stably expressing the RET/PTC(4F) or RET/PTC(3F) mutants, transfections were done by the calcium phosphate coprecipitation technique as described previously (11) and mass populations of several 10 cell clones were isolated for each transfection by G418 selection. Equal levels of expression of RET/PTC mutants were confirmed by immunoblotting. Transient transfections were carried out with the Lipofectamine reagent according to the manufacturer's instructions (Life Technologies).

Cell growth and staining. For growth curves, 0.5×10^5 cells were seeded in triplicate and counted at the indicated time points. DNA synthesis rate was measured with the 5'-bromo-3'-deoxyuridine (BrdUrd) Labeling and Detection kit from Boehringer Mannheim. Briefly, cells were seeded on glass coverslips, pulsed for 1 h with BrdUrd (final concentration of 10 μ mol/L), fixed, and permeabilized. Coverslips were incubated with anti-BrdUrd mouse monoclonal and rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and mounted in Moviol on glass slides. Cell nuclei were identified by Hoechst 33258 (final concentration, 1 μ g/mL; Sigma) staining. The fluorescent signal was visualized with an epifluorescent microscope (Axiovert 2, Zeiss; equipped with a $\times 100$ lens) interfaced with the image analyzer software KS300 (Zeiss). At least 100 cells were counted in five different microscopic fields; results were average fractions of BrdUrd-positive cells \pm SD.

Tissue samples. Archival frozen thyroid tissue samples from 12 patients affected by PTC (T1–T12) and 2 normal thyroids (N1–N2) were retrieved from the files of the Pathology Department of the University of Pisa. The study was approved by the Institutional Ethics Committee. Sections (4 μ m thick) of paraffin-embedded samples were stained with H&E for histologic examination to ensure that the samples met the diagnostic criteria required for the identification of PTC (enlarged nuclei with fine dusty chromatin, nuclear grooves, single or multiple micro/macro nucleoli, and intranuclear inclusions; ref. 27). According to a previous characterization, three of these PTC samples had a RET/PTC1 rearrangement (T2, T7, and T12) and six had a BRAF V600E mutation (T1, T4, T5, T6, T8, and T10; ref. 11). Snap-frozen tissue samples were kept in liquid nitrogen for storage at -80°C until protein extraction was done.

Plasmids. Unless otherwise specified, RET/PTC3 is the RET/PTC form used in this study. The RET/PTC3 constructs were cloned in pBABE and pCDNA3.1 (Invitrogen). They encode the short (RET-9) RET/PTC3 spliced form and are described elsewhere (11). For simplicity, we numbered the residues of RET/PTC proteins according to the corresponding residues in unarranged RET. Briefly, RET/PTC(K–) is a kinase-dead mutant carrying the substitution of the catalytic lysine (residue 758 in full-length RET) with a methionine. RET/PTC(4F) is a mutant in which the four autophosphor-

ylation sites (Y826, Y1015, Y1029, and Y1062) of the COOH-terminal tail are mutated to phenylalanines; in RET/PTC(3F), the Y1062 has been added back. Plasmids encoding the dominant-negative Ras(N17), MEK(DN), and AKT(DN); the constitutively active Ras(V12), BRAF(V600), and β -catenin (S374A); and the TCF/LEF reporter system are described elsewhere (28).

Antibodies and compounds. Anti-RET is an affinity-purified polyclonal antibody raised against the tyrosine kinase protein fragment of human RET. Anti- β -catenin (610153) and anti-E-cadherin (610181) were purchased from Becton Dickinson (BD Transduction Laboratories). Anti-phospho-p44/42 mitogen-activated protein kinase [MAPK; recognizing MAPK (ERK1/2) when phosphorylated either individually or dually on Thr202 and Tyr204], anti-p44/42 MAPK, anti-phospho-AKT (specific for AKT phosphorylated at Ser473), anti-AKT, anti-phospho-Ser21/9 GSK, anti-GSK, and anti-phospho- β -catenin (Ser33/37/Thr41) were purchased from Cell Signaling. Anti-tubulin was from Sigma. Monoclonal anti-phospho- β -catenin (Y654) antibody was from Abcam. Anti-cyclic AMP-responsive element binding protein (CREB), anti-phospho-S133 CREB (06-519), and anti-Tcf4 (05-511) were from Upstate Biotechnology, Inc. Anti-CREB-binding protein (CBP), anti-p300, anti-SP1, and anti-c-Myc antibody were from Santa Cruz Biotechnology. Secondary antibodies coupled to horseradish peroxidase were from Amersham Pharmacia Biotech. Cycloheximide was purchased from Sigma and used at 10 μ g/mL final concentration. LY294002 was from Calbiochem (Merck Chemicals Ltd.) and used at 10 μ mol/L final concentration. MAPK/ERK kinase (MEK)-1/2 inhibitor UO126 was from Cell Signaling and used at 10 μ mol/L final concentration.

Protein studies. Immunoblotting experiments were done according to standard procedures. Protein concentration was estimated with a modified Bradford assay (Bio-Rad). Immune complexes were detected with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Signal intensity was analyzed with the Phosphorimager (Typhoon 8600, Amersham Pharmacia Biotech) interfaced with the ImageQuant software. For immunoprecipitations, total lysates were incubated with 2 μ g antibody for 2 h at 4°C . Antibody-antigen complexes were collected with 30 μ L of protein G-Sepharose or protein A-Sepharose beads overnight at 4°C with gentle rotation. The samples were centrifuged, washed, eluted in sample buffer, and run on SDS-polyacrylamide gel. Nuclear extraction was done as described elsewhere (29). Briefly, cells were harvested in lysis buffer [10 mmol/L Tris-HCl (pH 7.9), 10 mmol/L KCl, 1.5 mmol/L MgCl_2 , 1 mmol/L DTT, 1 mmol/L phenylmethyl-sulfonylfluoride (PMSF), supplemented with 60 mmol/L NaF, 60 mmol/L β -glycerophosphate, and protease inhibitors (aprotinin, leupeptin, and pepstatin; 40 mg/mL)] and lysed by shearing with 15 passages through a 26-gauge needle mounted in a 1-mL syringe. Nuclei were recovered by centrifugation at $3,000 \times g$ for 10 min. Nuclear proteins were extracted in 50 mmol/L Tris-HCl (pH 7.5), containing 0.3 mol/L sucrose, 0.42 mol/L KCl, 5 mmol/L MgCl_2 , 0.1 mmol/L EDTA, 20% glycerol, 2 mmol/L DTT, 0.1 mmol/L PMSF, 60 mmol/L NaF, 60 mmol/L β -glycerophosphate, leupeptin, and aprotinin. Cytosolic fractions were recovered after membrane fraction removal by $100,000 \times g$ ultracentrifugation.

Pull-down assay. The GST-RET/PTC3 and GST-RET/TK plasmids were generated by PCR amplification of full-length RET/PTC3 or the isolated RET component of the RET/PTC3 protein (RET residues 718–1072) and fusion to the glutathione S-transferase (GST) coding sequence into the pEBG vector (kindly provided by S. Meakin, Laboratory of Neural Signaling, Cell Biology Group, The John P. Robarts Research Institute, London, Ontario, Canada; ref. 30). GST-RET/PTC3 and GST-RET/TK fusion proteins were purified from transiently transfected cell lysates using glutathione-Sepharose according to standard procedures. HEK293T cells were serum starved for 18 h and lysed in an ice-cold buffer. Protein lysates (2 mg) were incubated overnight with 30 μ g of GST-RET/PTC3 and GST-RET/TK fusion proteins. Pellet beads were collected by centrifugation ($14,000 \times g$) and washed with lysis buffer. The beads were resuspended in $2 \times$ Laemmli buffer and subjected to Western blotting.

Reporter assay. To evaluate the TCF/LEF transcriptional activity, we used a pair of luciferase reporter constructs, TOP-FLASH and the negative control FOP-FLASH (Upstate Biotechnology). TOP-FLASH contains three copies of the TCF/LEF binding site (AAGATCAAGGGGGT) upstream of

the thymidine kinase minimal promoter; FOP-FLASH contains a mutated TCF/LEF binding site (AAGGCCAAAGGGGT). Cells were transiently transfected by one of these reporters together with pRL-TK (encoding the Renilla luciferase) in triplicate, as instructed by the manufacturer (Promega Corporation). Luciferase activity was measured 48 h after transfection with the Dual-luciferase reporter assay system (Promega). TCF/LEF activity was determined by the TOP-FLASH/FOP-FLASH ratio after normalization for Renilla luciferase activity. Light emission was quantified with a Berthold Technologies luminometer (Centro LB 960). Data were represented as average fold change \pm SD with respect to the negative control.

RNA silencing. Predesigned duplex small interfering RNA (siRNA) against rat β -catenin (190086, 190087, and 190088) were provided by

Ambion. The scrambled oligonucleotide was synthesized by Prologo, and the sequence was 5'-AGGAUAGCGUGGAUUCGGUTT-3'. The day before transfection, cells were plated in six-well dishes at 30% to 40% confluency. Transfection was done using 5 μ g of duplex RNA and 6 μ L of Oligofectamine reagent (Invitrogen). Cells were harvested at 48 h posttransfection or counted at different time points for evaluating cell growth.

Chromatin immunoprecipitation. Chromatin was extracted from RET/PTC- or empty vector-transfected HEK293T cells. Chromatin immunoprecipitation assay was done by using the chromatin immunoprecipitation assay kit (Upstate Biotechnology), following the manufacturer's instructions. Chromatin was fixed by directly adding formaldehyde (1%, v/v) to the cell culture medium. Nuclear extracts were isolated and then

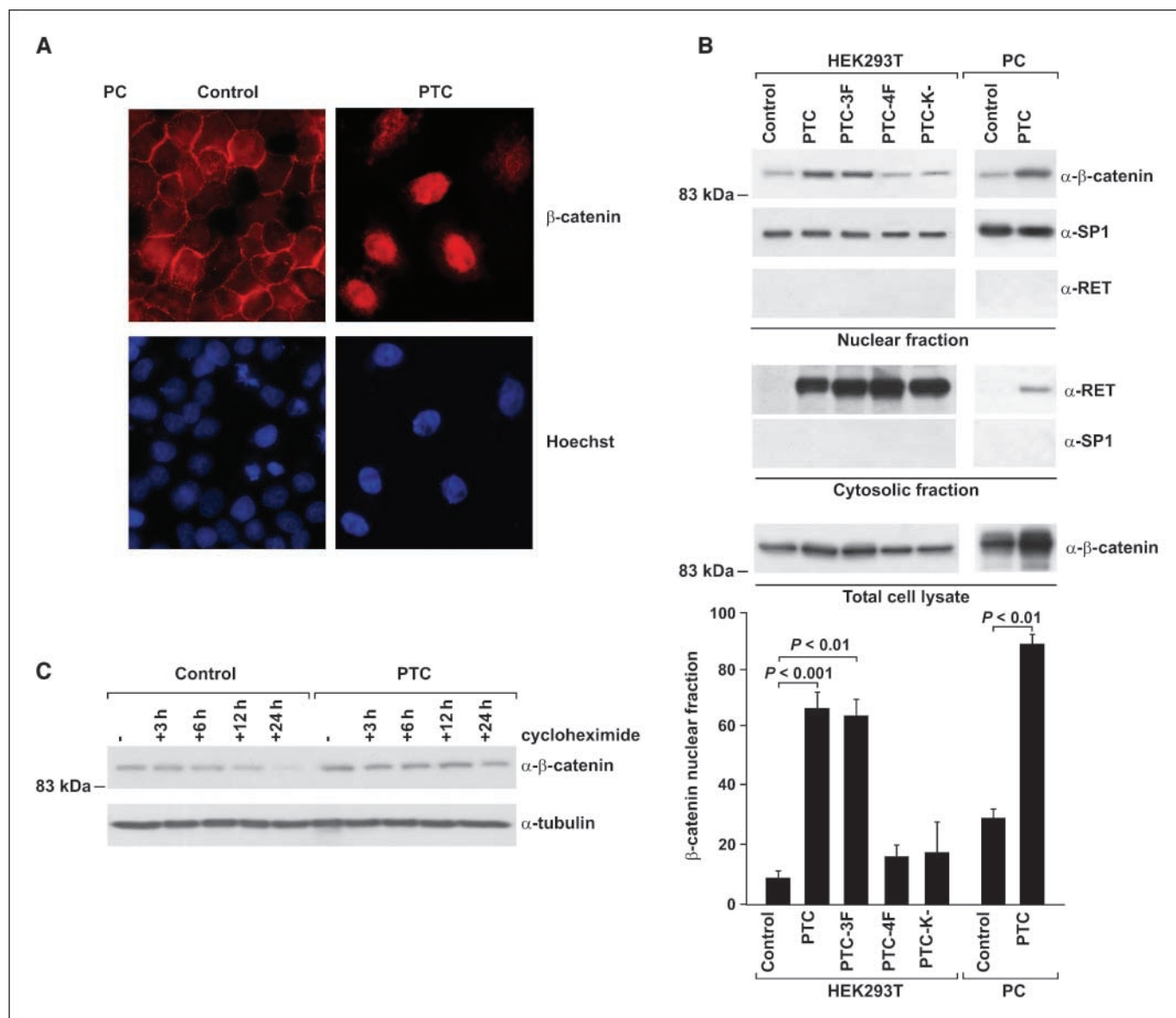


Figure 1. RET/PTC stabilizes β -catenin and promotes its nuclear accumulation. **A**, immunofluorescence for β -catenin in PC cells transiently transfected with RET/PTC. Note the increase of nuclear staining in transfected cells. Hoechst stain was used to visualize cell nuclei; parallel transfection with green fluorescent protein showed that \sim 20% of the cells expressed exogenous DNA (data not shown). **B**, nuclear translocation of β -catenin on RET/PTC expression. HEK293T cells were transfected with RET/PTC or the indicated mutants [mock transfected (Control)]. PC-RET/PTC cells or parental PC were also used. Subcellular fractions were obtained and nuclear extracts (50 μ g) probed with anti- β -catenin. The purity of the fractions was assessed by verifying the absence of SP1 in the cytosolic fraction and of RET/PTC in the nuclear fraction. Total β -catenin levels were also measured in unfractionated extracts. Columns, mean percentage of nuclear β -catenin with respect to total levels from five independent determinations; bars, SD. Reported P values were calculated by Student's t test. **C**, HEK293T cells transiently expressing RET/PTC or a control vector were treated with cycloheximide for different time points. Lysates (30 μ g) were run on SDS-PAGE, and β -catenin levels were determined by immunoblot.

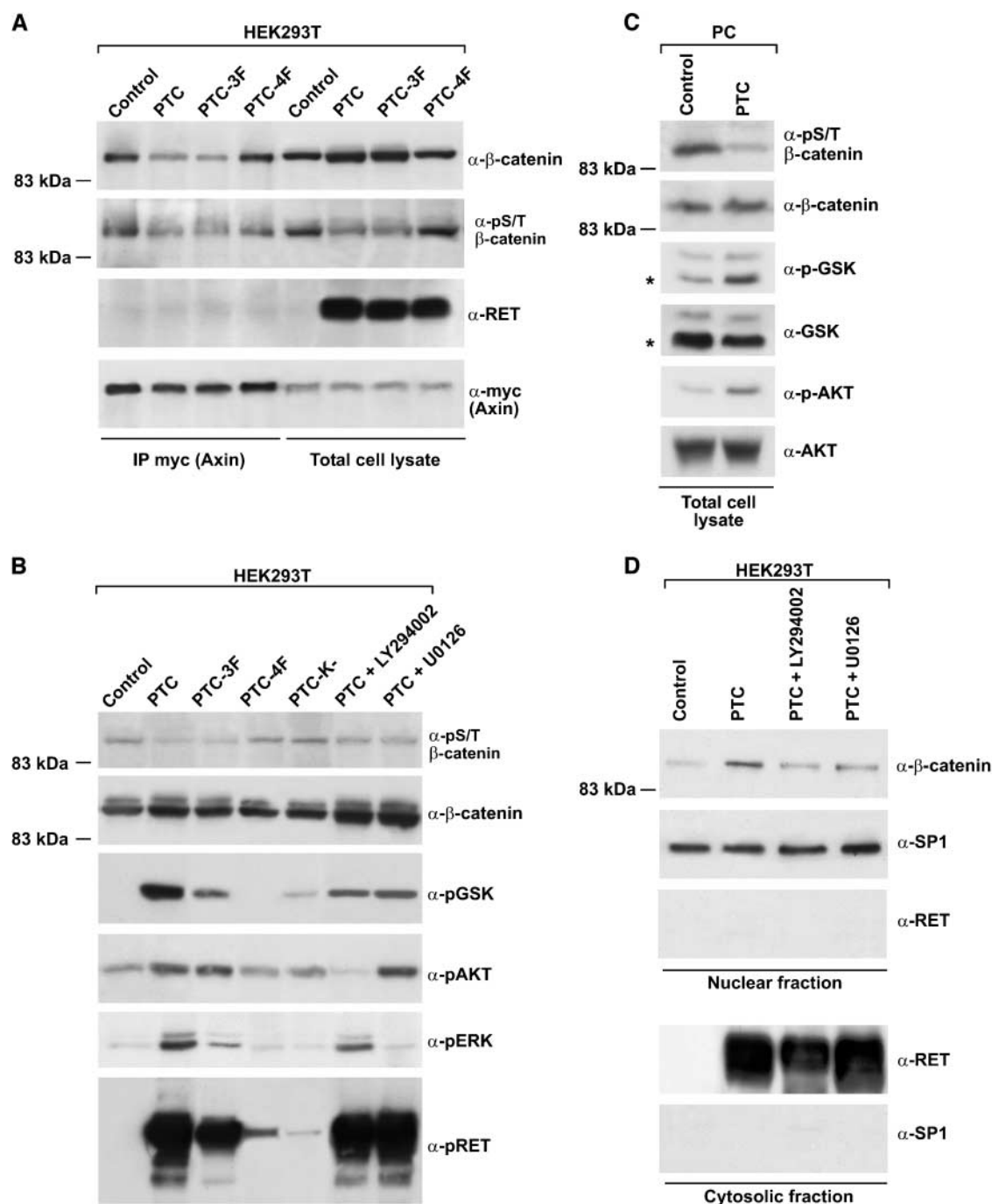
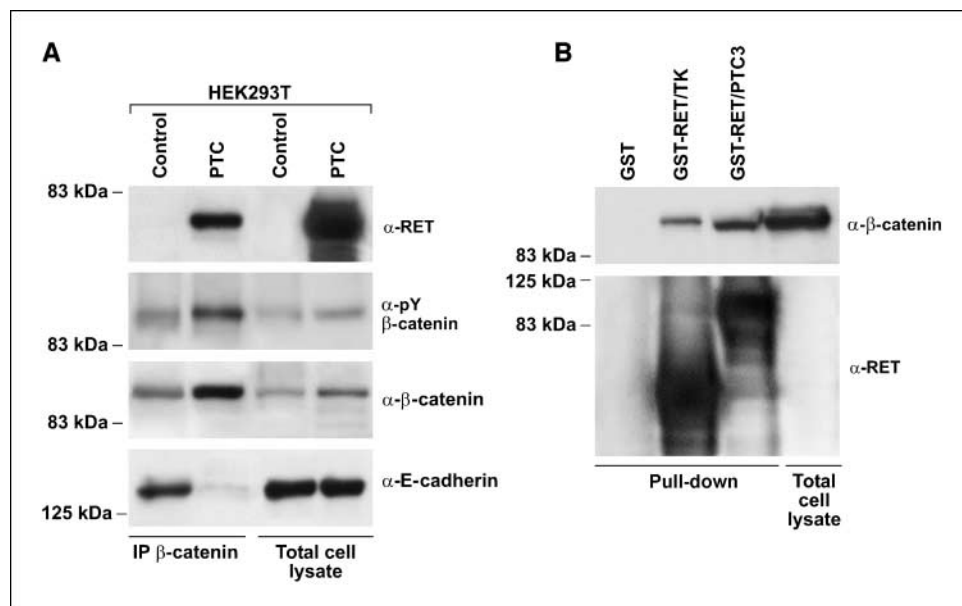


Figure 2. RET/PTC induces the dissociation of the β -catenin degradation complex. **A**, HEK293T cells were transfected with a myc-tagged Axin together with RET/PTC or the indicated mutants. Protein complexes containing Axin were recovered by immunoprecipitation (1 mg) with anti-myc tag and blotted against β -catenin or S/T-phosphorylated β -catenin (phospho-Ser33/37/Thr41). Myc-Axin and RET/PTC protein levels in total cell lysates are shown for normalization. **B** and **C**, phosphorylation levels of GSK3 β , β -catenin (phospho-Ser33/37/Thr41), AKT, ERK, and RET (Y905) were measured by immunoblot of total cell lysates (50 μ g) harvested from HEK293T (**B**) or PC (**C**) cells expressing the indicated RET/PTC variants or treated with the indicated compounds. GSK3 β antibodies may recognize both GSK-3 α (51 kDa) and GSK3 β (46 kDa) proteins; *, GSK3 β migration (**C**). **D**, nuclear accumulation of β -catenin (measured as in Fig. 1B) in HEK293T cells transfected with RET/PTC and treated or not with chemical PI3K or MEK inhibitors. Representative data of at least three independent experiments.

fragmented through sonication. Transcription factor-bound chromatin was immunoprecipitated with β -catenin, CREB, or TCF antibodies; cross-linking was reversed; and the isolated genomic DNA amplified by quantitative PCR using primers spanning either the CRE site (Fig. 4D) or the TCF/LEF

binding site (data not shown) of the human cyclin D1 promoter: CRE-forward, 5'-AACGTCACACGGACTACAGG-3'; CRE-reverse, 5'-TGTTCATGGCTGGGGCTCTT-3'; TCF-forward, 5'-GAGCGCATGC-TAAGCTGAAA-3'; TCF-reverse, 5'-GGACAGACGGCCAAAGAATC-3'.

Figure 3. RET/PTC interaction with β -catenin. **A**, protein lysates (2 mg) from mock-transfected or RET/PTC-transfected HEK293T cells were immunoprecipitated with anti- β -catenin and probed with tyrosine-phosphorylated β -catenin (pY654), RET, or E-cadherin antibodies. Expression levels in total cell lysates are shown for normalization. **B**, full-length RET/PTC3 and the isolated RET component of the RET/PTC3 (deprived of the NCOA4 region) protein were produced as GST-fusion proteins and used to pull down β -catenin from HEK293T protein lysates. Immunoblot was stained with anti- β -catenin; recombinant RET proteins input is also reported.



Fluorescent threshold values (C_t) were measured in triplicate for immunoprecipitated samples as well as for an aliquot of the input DNA. Results were calculated by the following formula: $2^{-[\Delta C_t/C_t \text{ input DNA}]}$, where ΔC_t is the difference between the C_t of the specific antibody-immunoprecipitated DNA and the C_t of mock-immunoprecipitated DNA. Results are average \pm SD of triplicate samples.

Statistical analysis. Student's t test was used for statistical analysis. All P values were two sided and differences were significant when $P < 0.05$.

Results

RET/PTC promotes the nuclear accumulation of β -catenin. Thyroid follicular PC cells transiently transfected with RET/PTC accumulated nuclear β -catenin, a hallmark of β -catenin activation (Fig. 1A). By biochemical fractionation, although total β -catenin levels were increased on RET/PTC expression, nuclear β -catenin levels were proportionally increased to a greater extent, thereby accounting for the increase in the nuclear β -catenin fraction in HEK293T or PC cells [7-fold ($P < 0.001$) or 3.6-fold ($P < 0.01$), respectively] on transient or stable RET/PTC expression (Fig. 1B). β -Catenin nuclear accumulation depended on RET/PTC kinase activity because it was reduced by a kinase-dead mutant (K-). The residual, albeit not significant, activity of PTC(K-) might depend on RET kinase rescue operated by other tyrosine kinases, such as epidermal growth factor receptor, as recently shown (31). Moreover, β -catenin accumulation depended on RET/PTC autophosphorylation because it was not exerted by a RET/PTC mutant (PTC-4F) whose major autophosphorylation sites (Y826, Y1015, Y1029, and Y1062) were mutated to phenylalanine (Fig. 1B). Y1062 was essential because nuclear accumulation of β -catenin was restored when tyrosine Y1062 was added back to the 4F mutant (PTC-3F; $P < 0.01$; Fig. 1B). There was no detectable difference in the mRNA levels of β -catenin between RET/PTC-positive and RET/PTC-negative cells, which suggests that the increase in β -catenin occurred at the posttranscriptional level (data not shown). Accordingly, the half-life of β -catenin increased in RET/PTC-expressing cells (>24 hours) versus control HEK293T (~12 hours; Fig. 1C).

RET/PTC targets the Axin-GSK3 β - β -catenin complex. The pathway leading to β -catenin activation involves a series of events that result in the dissociation of β -catenin from Axin, a scaffold protein that forms a large molecular complex with APC, Dsh, and GSK3 β (17, 18). Transient RET/PTC expression in HEK293T cells decreased the amount of β -catenin coprecipitating with myc-tagged Axin; this effect depended on Y1062, as shown when we used the PTC-4F mutant (Fig. 2A). Secondary to the assembly of the Axin-GSK3 β - β -catenin complex, phosphorylation of β -catenin by GSK3 β in NH₂-terminal Ser33, Ser37, and Thr41 promotes its ubiquitin-dependent proteolytic degradation (19–22). RET/PTC expression in HEK293T reduced amounts of S/T-phosphorylated β -catenin coprecipitating with Axin (Fig. 2A). Moreover, transient RET/PTC expression in HEK293T (Fig. 2A and B) and stable expression in PC (Fig. 2C) reduced overall S/T β -catenin phosphorylation by immunoblot, an effect that depended on RET/PTC kinase and Y1062 (Fig. 2B). β -Catenin phosphorylation is affected by GSK3 β . In turn, the activity of GSK3 β can be blocked by both AKT- (19) and ERK pathway-mediated phosphorylation at Ser9 (22). RET/PTC stimulates both the PI3K/AKT and the ERK pathways by recruiting several adaptors to phosphorylated Y1062 (2). RET/PTC-triggered phosphorylation of AKT and ERK paralleled the phosphorylation of GSK3 β -Ser9 and the consequent reduction of phospho-S/T β -catenin (Fig. 2B and C). PTC-4F had a significantly reduced activity with respect to wild-type RET/PTC (Fig. 2B). In addition, PTC-3F (although expressed at lower levels) had a reduced effect on GSK3 β phosphorylation, suggesting that COOH-terminal tyrosines other than Y1062 may participate to this pathway. LY294002, a PI3K inhibitor, partially impaired GSK3 β phosphorylation and increased β -catenin S/T phosphorylation; these effects were associated with a reduction in AKT phosphorylation (Fig. 2B). In addition, treatment with U0126, a MEK inhibitor, reduced phosphorylation of GSK3 β and partially rescued phospho-S/T β -catenin (Fig. 2B). Finally, treatment with LY294002 or U0126 reduced nuclear accumulation of β -catenin (Fig. 2D).

RET/PTC increases β -catenin phosphotyrosine content. β -Catenin interacts directly with the oncogenic tyrosine kinases c-src

(32), c-MET (33), RON (34), c-erbB-2 (35), and BCR-ABL (36), thereby resulting in β -catenin phosphorylation on tyrosine residues. More importantly, Gujral and colleagues (25) recently reported that MEN2-associated RET point mutants bind to β -catenin and phosphorylate it on tyrosine 654. We tested whether this mechanism was also used by RET/PTC. In HEK293T cells, RET/PTC coimmunoprecipitated with β -catenin (Fig. 3A), and on RET/PTC expression, tyrosine-phosphorylated β -catenin (pY654) increased; overall increased β -catenin levels may partially account for these effects (Fig. 3A). The binding of β -catenin to RET/PTC paralleled its dissociation from E-cadherin (Fig. 3A). Moreover, recombinant GST-RET/PTC3 and GST-RET/TK proteins were able to pull down β -catenin from HEK293T cell lysates, showing an interaction, either direct or mediated by intermediate protein(s), between the RET component of RET/PTC and β -catenin (Fig. 3B).

RET/PTC stimulates TCF/LEF- and CREB-mediated cyclin D1 transcription. Nuclear β -catenin forms complexes with members of the TCF and LEF family of DNA-binding proteins, and this process results in activation of target gene promoters. RET/PTC stimulated the activity of a luciferase TCF/LEF-dependent reporter gene system (TOPflash) in both HEK293T and PC cells ($P < 0.01$; Fig. 4A). The mutant TOPflash reporter (FOPflash), bearing a mutated TCF/LEF site, was used to subtract background. This activity depended on the integrity of the RET/PTC kinase and of the Y1062 multidocking site ($P < 0.05$; Fig. 4A, left). Again, other tyrosines besides Y1062 likely played a role because the PTC-3F mutant was impaired with respect to wild-type RET/PTC (Fig. 4A, left). TOPflash expression was partially reduced by treatment with LY294002 and U0126 and by the coexpression of dominant negative mutants for Ras (N17), MEK (MEKDN), and AKT (AKTDN; $P < 0.05$). Constitutively active BRAF (V600E) and Ras (V12) stimulated the TCF/LEF reporter in HEK293T cells, although to a lesser extent than RET/PTC. A transcriptionally active form of β -catenin (S374A) served as a positive control (Fig. 4A).

Besides TCF/LEF, β -catenin recruits the activated form (serine 133 phosphorylated) of CREB, thereby resulting in a transcriptionally active complex, which in turn binds CBP/p300 (37–40). TCF/LEF and CREB collaborate in regulating the transcription of TCR α (41) and WISP-1 (42). Finally, RET signaling through Y1062 and Ras/ERK increases S133 phospho-CREB levels (2). To determine whether RET/PTC affected the binding of CREB to β -catenin, we immunoprecipitated β -catenin from PC cells in the presence or absence of RET/PTC and probed the immunocomplexes with anti-CREB and anti-CBP/p300 antibodies. Expression of RET/PTC significantly increased the amounts of β -catenin bound to CREB and CBP/p300 (Fig. 4B). Chemical blockade of MEK by U0126 reduced this association, and this effect was paralleled by a reduction in CREB phosphorylation on Ser133 and ERK phosphorylation (Fig. 4B). In a mirror experiment, RET/PTC expression increased not only TCF/LEF- β -catenin (Fig. 4C, bottom) but also CREB- β -catenin protein complexes (Fig. 4C, top). In HEK293T cells, formation of both complexes was reduced when kinase-dead and 4F mutants were used (Fig. 4C, right). Instead, by immunoprecipitating CREB and staining with TCF/LEF, we did not detect TCF/LEF-CREB interaction, suggesting that β -catenin-containing TCF/LEF and CREB complexes are distinct (Fig. 4C, middle).

Cyclin D1 promoter contains adjacent TCF/LEF (at –81 bp) and CREB (at –58 bp) binding sites (40). We used chromatin

immunoprecipitation to measure β -catenin, TCF/LEF, and CREB binding to the region of the cyclin D1 promoter that contains CREB and TCF/LEF binding sites. For PCR, we used two primer pairs spanning the CRE (Fig. 4D) and TCF (data not shown) sites, respectively. With both primer sets, binding of β -catenin, TCF/LEF, and CREB to the cyclin D1 promoter was greatly increased on RET/PTC expression ($P < 0.05$; Fig. 4D). Because the binding sites for CREB and TCF/LEF are just a few nucleotides apart on the cyclin D1 promoter, this experiment left open possibilities that the two β -catenin-containing (CREB and TCF/LEF) complexes can be either identical or distinct; however, the lack of coimmunoprecipitation in Fig. 4C favors the possibility that the two complexes are distinct.

RET/PTC-mediated mitogenic signaling depends on β -catenin. In PC cells, RET/PTC-mediated increase in β -catenin activity was paralleled by accumulation of the TCF/LEF and CREB target protein cyclin D1 and, more weakly, c-Myc (Fig. 5A). We knocked down β -catenin by RNA interference in parental and RET/PTC-expressing PC cells. As shown in Fig. 5A, β -catenin-specific siRNA, but not the scrambled control, reduced the expression of β -catenin by >50% in both PC and PC-PTC cells. Moreover, β -catenin siRNA reduced cyclin D1 and, more weakly, c-Myc levels in RET/PTC-expressing cells. β -Catenin RNA interference reduced hormone-independent proliferation of PC-PTC cells (Fig. 5B, bottom), whereas a constitutively active form of β -catenin (S374A) stimulated hormone-independent growth of PC cells (Fig. 5B, top). We generated mass populations of PTC-4F- and PTC-3F-expressing PC cells. On hormone deprivation, we counted S-phase cells on a 1-hour BrdUrd pulse after transient transfection with scrambled or β -catenin specific siRNA. Similarly to wild-type PTC, PTC-3F-expressing, but not PTC-4F-expressing, cells incorporated BrdUrd in the absence of hormones in a β -catenin-dependent manner ($P < 0.01$). Taken together, these findings show that β -catenin is a mediator of the RET/PTC mitogenic signaling in thyrocytes.

Finally, to study the β -catenin pathway in human PTC samples, we measured levels of GSK3 β -Ser9 and S/T β -catenin phosphorylation compared with normal thyroid samples ($n = 2$) in a small set of PTC ($n = 12$) samples (three RET/PTC positive, six BRAF V600E positive, and the remainder RET/PTC and BRAF negative). Reduced levels of β -catenin S/T phosphorylation was visible in virtually all the tumor samples; increased levels of GSK3 β -Ser9 phosphorylation and total β -catenin were detectable in ~75% and 50% of them, respectively (Fig. 5D, left). Similarly, thyroid carcinoma cell lines positive either for RET/PTC1 (TPC-1) or for BRAF V600E (BCPAP, 8505C, and OCUT-1) showed reduced levels of S/T β -catenin and increased GSK3 β -Ser9 phosphorylation when compared with a primary culture of normal thyrocytes (Fig. 5D, right).

Discussion

Here we describe the functional interaction between the RET/PTC and the β -catenin signaling pathways. β -Catenin activation by RET/PTC occurred through several coordinated mechanisms (Fig. 6). As previously reported by Gujral and colleagues (25) for point mutant RET, RET/PTC induced the tyrosine phosphorylation of β -catenin, thereby mobilizing the fraction of β -catenin associated to E-cadherin and increasing its free cytosolic pool. RET/PTC-mediated activation of PI3K/AKT and Ras/ERK contributed to promote β -catenin stabilization through inactivation of

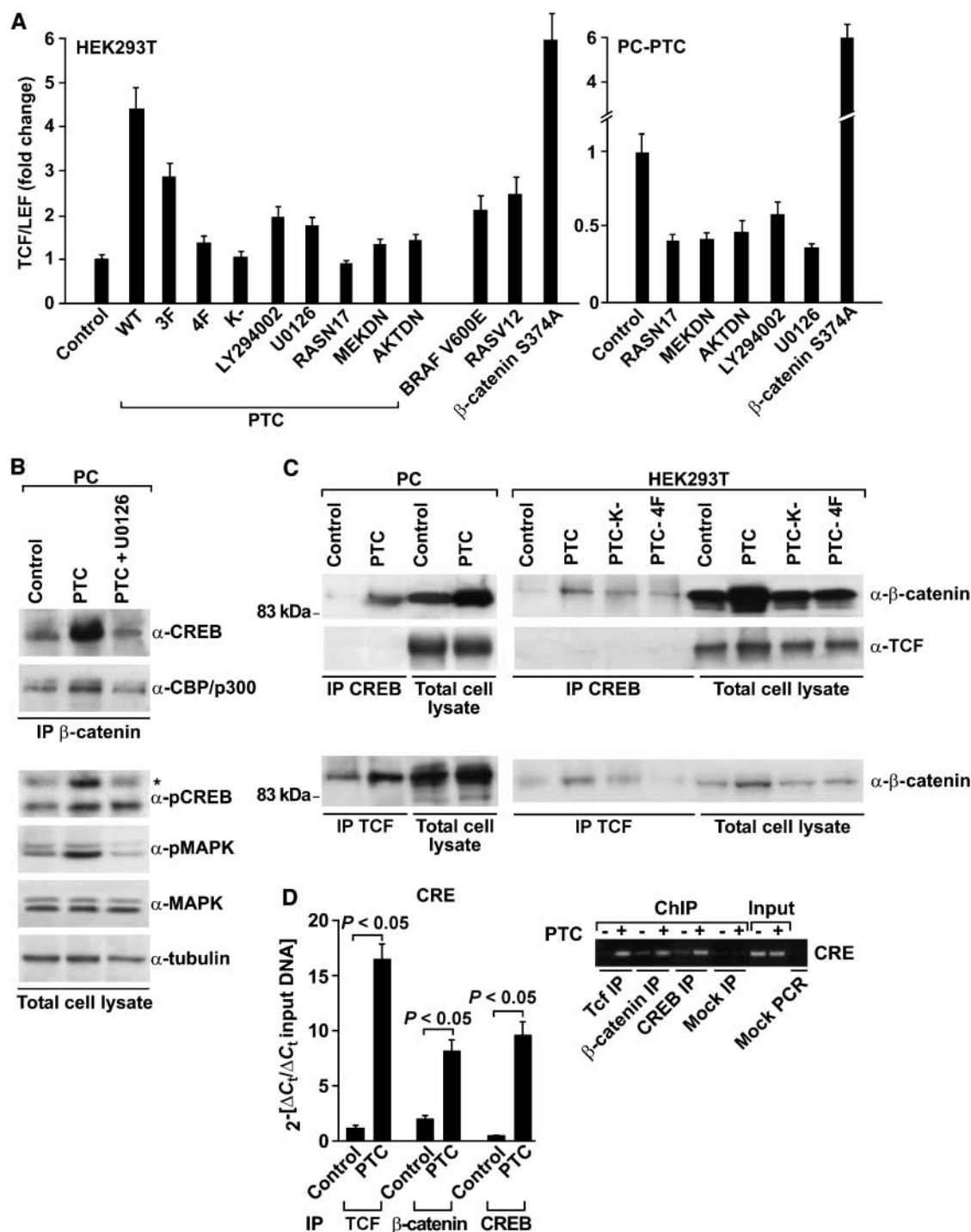


Figure 4. RET/PTC-mediated activation of TCF/LEF and CREB transcription factors. **A**, HEK293T (*left*) and PC-PTC cells (*right*) were transfected with the indicated plasmids together with the TOPflash reporter and, when indicated, treated with the chemical inhibitors. Luciferase activity was expressed as fold increase with respect to mock-transfected cells. *Columns*, average results of three independent assays; *bars*, SD. Reported *P* values were calculated by Student's *t* test. **B**, cellular lysates (1 mg) from PC and PC-PTC cells, treated or not with U0126, were immunoprecipitated with anti-β-catenin and probed with CREB and CBP/p300 antibodies. The CREB antibody may also recognize, besides CREB (43 kDa), CREM (30 kDa) and activating transcription factor-1 (38 kDa); *, CREB migration. Phosphorylation of CREB (S133) and ERK (MAPK) was measured in total cell lysates by immunoblot. Tubulin was used for normalization. **C**, lysates from PC or PC-PTC cells (*left*) and HEK293T cells transiently expressing the indicated RET/PTC constructs (*right*) were immunoprecipitated with CREB and TCF antibodies and probed for β-catenin or TCF antibodies, as reported. **D**, chromatin immunoprecipitation (*ChIP*) was done in HEK293T cells transfected with RET/PTC or the empty vector (*control*). Processed DNA was immunoprecipitated with TCF, β-catenin, or CREB antibodies and subjected to real-time PCR (*bar graphs*) with amplimers spanning the CRE site of the cyclin D1 promoter. *Columns*, mean of triplicate samples; *bars*, SD. Fluorescent threshold values (*C_t*) were measured for immunoprecipitated samples as well as for an aliquot of the input DNA. Reported *P* values were calculated by Student's *t* test. Semiquantitative PCR (ethidium bromide stain) was also done with the same primers by using 1 μL of the DNA and 25 cycles of amplification. Amplification of the expected fragments in the input samples indicated equal input; mock PCR was done without template.

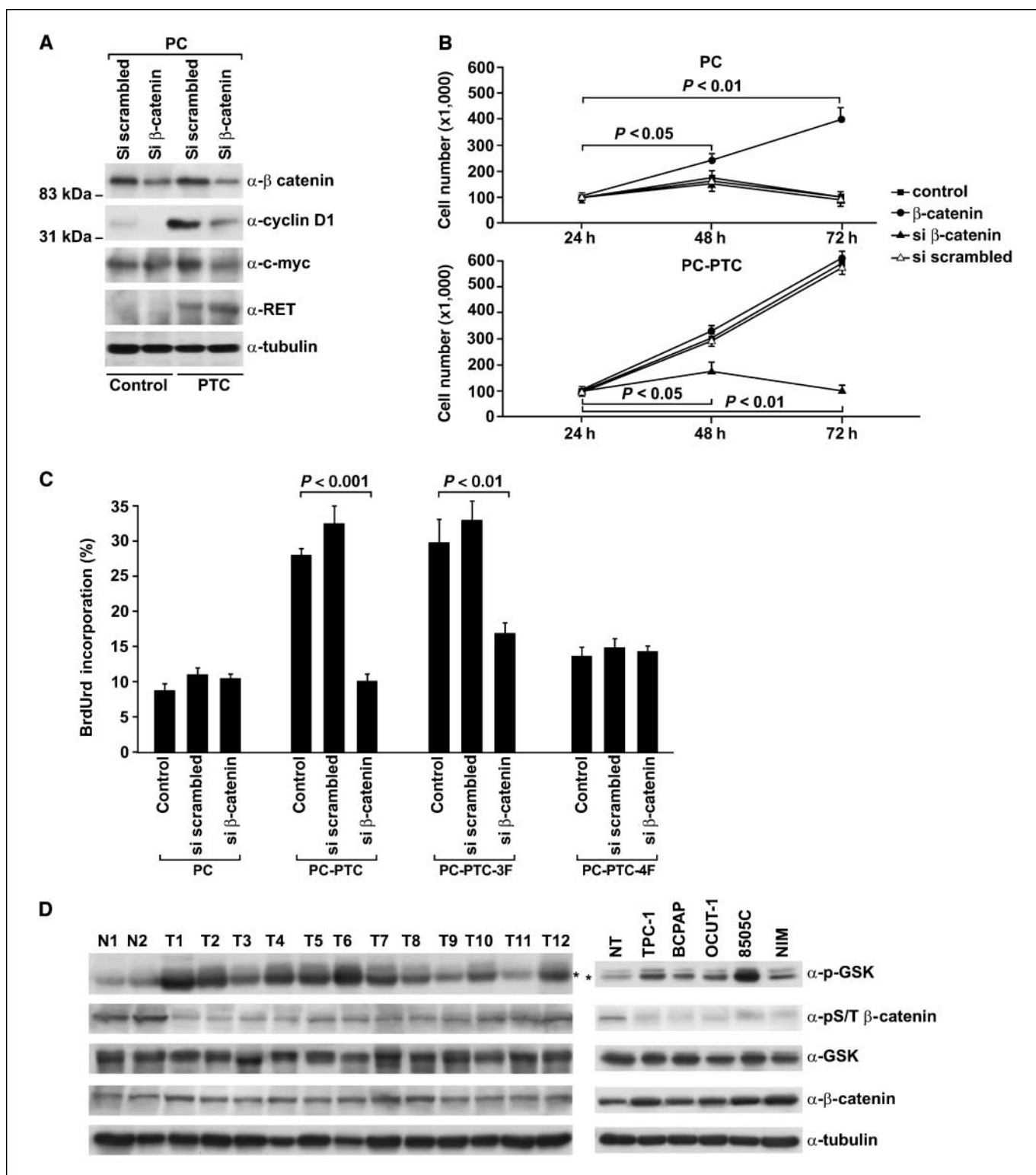
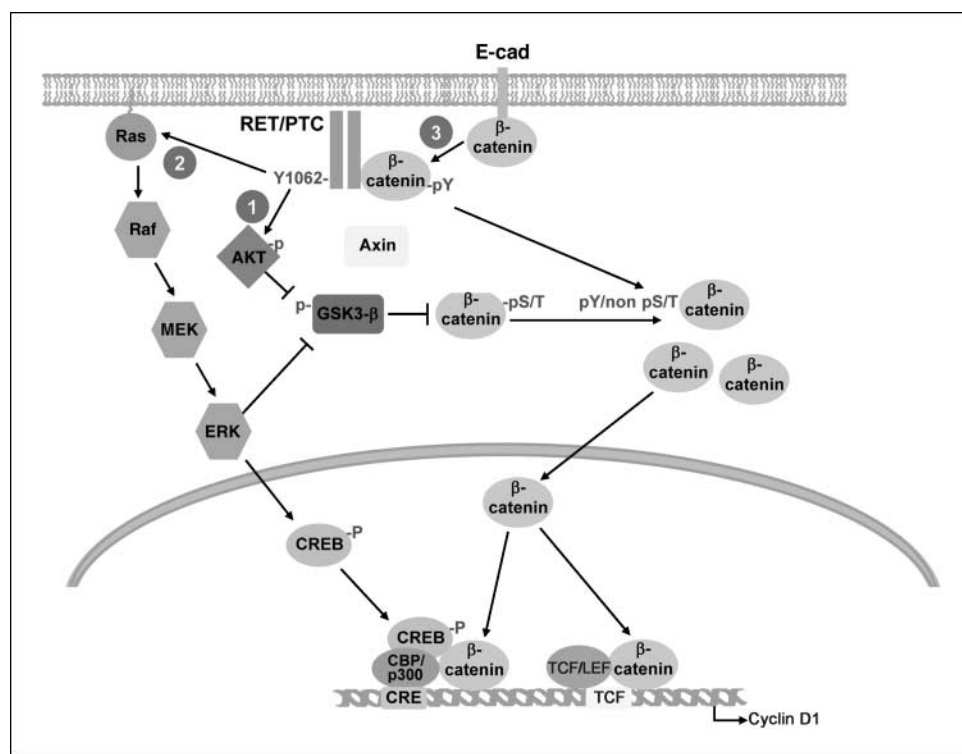


Figure 5. β -Catenin contributes to autonomous growth of RET/PTC-expressing thyroid cells. **A**, β -catenin was knocked down by transient transfection with specific siRNA (*si β-catenin*) in parental or RET/PTC-expressing PC cells. As a control, cells were treated with scrambled siRNA (*si scrambled*). Protein levels were measured by immunoblot with the indicated antibodies. **B**, proliferation in the absence of hormones of parental, constitutively active β -catenin (S374A) or β -catenin siRNA transiently transfected PC or PC-PTC cells was measured by cell counts. Points, mean of triplicate samples; bars, SD. Reported *P* values were calculated by Student's *t* test. **C**, mass populations of PTC-4F- and PTC-3F-expressing PC cells were generated by stable transfection and marker selection. On hormone deprivation and transfection with scrambled or β -catenin-specific siRNA (as in **A**), cells were pulsed (1 h) with BrdUrd, and BrdUrd-positive cells were analyzed by immunofluorescence. At least 100 cells were counted in five different microscopic fields. Columns, mean fractions of BrdUrd-positive cells; bars, SD. Reported *P* values were calculated by Student's *t* test. **D**, phosphorylation levels of GSK3 β and β -catenin (phospho-Ser33/37/Thr41) were measured by immunoblot of total cell lysates (50 μ g) harvested from PTC (T1–T12) and normal thyroid (N1–N2) tissue samples (left) or thyroid carcinoma and normal human primary cells (NT; right). GSK3 β antibodies may recognize both GSK3 α (51 kDa) and GSK3 β (46 kDa) proteins; *, GSK3 β migration.

Figure 6. A summary of the pathways leading to β -catenin induction by RET/PTC. 1 and 2, RET/PTC activates PI3K/AKT and Ras/MEK/ERK. This leads to GSK3 β phosphorylation, thereby relieving its negative control on β -catenin, and to an increase of the free β -catenin protein pool. 2, through ERK activation, RET/PTC induces S133 phosphorylation of CREB, which associates in a complex with β -catenin and CBP/p300 to stimulate transcription of cyclin-D1. 3, RET/PTC directly binds β -catenin and increases its phosphotyrosine content, thereby decreasing the E-cadherin-bound pool and increasing the free β -catenin pool. Free β -catenin enters the nucleus and participates in at least two different transcription complexes with CREB/p300 and with TCF/LEF, both ultimately involved in the regulation of cyclin D1.



GSK3 β . However, neither pathway was probably sufficient because chemical blockade of either one reduced but did not abrogate β -catenin accumulation. Finally, RET/PTC signaling favored the formation of transcriptional complexes containing β -catenin by triggering ERK-mediated CREB phosphorylation. Ser133-phosphorylated CREB formed a protein complex with CBP and β -catenin, which participated in the transcription of cyclin D1. TCF/LEF and CREB binding sites coexist in the promoters of some β -catenin target genes, such as *cyclin D1* (40), *TCRx* (41), and *WISP-1* (42), and collaborate in their firing. Accordingly, our findings suggest that downstream of RET/PTC, β -catenin participates in two distinct transcriptional complexes, one with TCF/LEF and another one with CREB, and that both are recruited to cyclin D1 promoter. The particularly important role played by Y1062 in signaling to β -catenin in the case of RET/PTC with respect to full-length RET mutants (25) may be explained by differential signaling mechanisms between the distinct RET oncogenic forms and the cytosolic localization of RET/PTC that relies on Y1062 to be recruited to the cell membrane (2–4). Finally, β -catenin expression is required for RET/PTC-mediated autonomous proliferation of PC thyrocytes.

It is possible that β -catenin activity cooperates with other signaling cascades activated by RET/PTC to mediate mitogenic activity. Cytosolic β -catenin is recruited to a “destruction” complex that includes APC and GSK3 β . Loss of APC tumor suppressor reduces the activity of the β -catenin destruction complex (17, 21). Of direct relevance to thyroid tumorigenesis, patients affected by familial APC [familial adenomatous polyposis (FAP)] and harboring APC mutations are predisposed to thyroid carcinoma (43). Furthermore, the RET/PTC oncogene is activated in some FAP-associated thyroid carcinomas (44). Therefore, our data support a model whereby two different lesions concomitantly present in FAP-associated thyroid carcinoma (i.e., RET/PTC

activation and APC loss of function) may converge to enhance the activity of the β -catenin signaling cascade. On the other hand, direct β -catenin mutations are restricted to aggressive and undifferentiated types of thyroid carcinomas (23).

β -Catenin targeting approaches for cancer therapy are currently being explored. For example, a conditionally replicative adenovirus, which kills only cells with a hyperactive β -catenin pathway, significantly inhibited the growth of undifferentiated (anaplastic) thyroid cancers (45). ICG-001, an inhibitor of the β -catenin/CBP transcriptional complex, efficiently induced apoptosis of colon cancer cells (46). Prostaglandin E₂ (PGE₂) also promotes β -catenin signaling (28), and PGE₂ synthesis inhibitors, nonsteroidal anti-inflammatory drugs, in an adjuvant or preventive setting are being tested already in cancer. Our data, together with those reported by Gujral and colleagues (25), suggest that β -catenin targeting approaches could have therapeutic potential in thyroid cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Attached Manuscript #II

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ORIGINAL ARTICLE

A novel *de novo* germ-line V292M mutation in the extracellular region of RET in a patient with pheochromocytoma and medullary thyroid carcinoma: functional characterization

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Summary

Context In multiple endocrine neoplasia (MEN), rearranged during transfection (*RET*), gene testing has been extensively exploited to characterize tumour aggressiveness and optimize the diagnostic and clinical management.

Objective To report the underlying genetic alterations in an unusual case of MEN type 2 (MEN-2A).

Design and patient Occult medullary thyroid carcinoma (MTC) was diagnosed in a 44-year-old man who had presented with unilateral pheochromocytoma. DNA extracted from the blood and tumour tissues was analysed for mutations in *RET*. The transforming potential and mitogenic properties of the identified *RET* mutation were investigated.

Results The patient carried a novel heterozygous germ-line *RET* mutation in exon 5 (Val292Met, GTG>ATG) (V292M/*RET*) with no evidence of additional somatic alterations. The mutation maps to the third cadherin-like domain of *RET*, which is usually not included in *RET* screening. Interestingly, MTC with concomitant pheochromocytoma has never been associated with a *RET* mutation involving the extracellular cadherin-like domain. V292M/*RET* was absent in the only two relatives examined. *In vitro* assays indicate that the mutant has low-grade transforming potential.

Conclusions Complete characterization and classification of all novel *RET* mutations are essential for extending genetic analysis in clinical practice. Our findings suggest that: (i) in all MEN-2 patients negative for *RET* hot-spot mutations, testing should be extended to all coding regions of the gene and (ii) the newly identi-

fied V292M/*RET* mutation is characterized by relatively weak *in vitro* transforming ability.

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Introduction

Genetic testing is a cornerstone of medullary thyroid carcinoma (MTC) management,^{1–3} and screening for germ-line mutations in the rearranged during transfection (*RET*) proto-oncogene⁴ is now done routinely in multiple endocrine neoplasia type 2 (MEN-2) patients and their relatives.⁵ This approach has revolutionized the management of MTC by allowing preclinical identification of family members at risk. Knowledge of the specific mutation can also guide clinical decisions, including those regarding the timing of prophylactic thyroidectomy, which significantly improves the prognosis, and intraoperative management of the cervical lymph nodes and parathyroid glands.^{6–8} Equally important, *RET* testing also spares noncarriers the ordeal and anxiety of yearly surveillance for cancer. Optimal interpretation of test results naturally requires thorough knowledge of each mutation, its relative tumourigenic potency, and its precise phenotypic correlates.

Most laboratories focus on the hot-spot *RET* exons (10, 11, 13, 14, 15 and 16), where the vast majority of the reported mutations are found.^{6,9–11} This approach is generally cost-effective although MEN-2A (the most common form of the MEN-2 syndrome) is occasionally associated with mutations in other *RET* regions.⁵ A few families have also been found to be negative for all known *RET* mutations, suggesting that predisposition to MEN-2 may also be caused by mutations at other loci.¹²

We report a patient with a MEN-2A phenotype consisting of unilateral pheochromocytoma (the presenting manifestation of the

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disease) and MTC. Genetic studies ultimately revealed a germ-line *RET* mutation in exon 5, which has not been previously described. *In vitro* experiments were performed to characterize its biological activity and relevance to the MEN-2A phenotype.

Materials and methods

All subjects provided written, informed consent to genetic studies and to publication of the findings reported later. Commercial products were used according to manufacturers' instructions unless otherwise stated.

Patient description

A 44-year-old man presented with a 2-year history of paroxysmal headaches associated with sweating, palpitations, tremor, dizziness, hypertension (240/110 mmHg). Physical examination was unremarkable, but biochemical evaluation revealed elevated urinary catecholamines (Table 1). Computed tomography disclosed a large right adrenal mass, which later displayed high uptake of metaiodobenzylguanidine. Serum calcium and phosphate levels were normal. The basal serum calcitonin level was normal. Neck ultrasonography disclosed a hypoechoic nodule (6 mm) with microcalcifications in the upper third of the right thyroid lobe, which presented at ultrasound-guided fine needle aspiration cytological findings suggestive of thyroid cancer.

The right adrenal tumour was surgically resected and histologically diagnosed as pheochromocytoma. After surgery, urinary catecholamine levels normalized, and the hypertensive episodes ceased. Six weeks later, total thyroidectomy was performed with dissection of four lymph nodes. Histology revealed the presence of a 4-mm unifocal MTC, with typical immunostaining features (calcitonin positivity, thyroglobulin negativity). In addition, foci of C cell hyperplasia were also present in both thyroid lobes. All four resected lymph nodes were negative for metastatic infiltration.

DNA sequence analysis

RET exons 5, 8, 10, 11, 13, 14, 15, 16 and 18 were analysed by direct sequencing in DNA extracted from peripheral blood (QIAamp-Blood MIDI kit; Qiagen, Valencia, CA, USA) and formalin-fixed, paraffin-embedded tumour tissues (Nucleon HT kit; Amersham Pharmacia Biotech, Milan, Italy) (For primers see Table 2). In DNA from the pheochromocytoma, we also sequenced all exons of the von Hippel-Lindau (*VHL*) gene and the genes encoding succinate

dehydrogenase subunits B (SDHB) and D (SDHD). The coding regions and exon-intron boundaries of each gene were amplified by PCR and the products sequenced with a BigDye Terminator version 3.1 Cycle Sequencing kit in an automated 3130xl analyzer (both from Applied Biosystems, Foster City, CA, USA).

Protein studies

Polyclonal rabbit antibodies against RET's tyrosine kinase domain (amino acids 738–1058) (anti-RET) or RET proteins phosphorylated on tyrosines 1062 and 905 (anti-pY1062 and anti-pY905, respectively) were affinity-purified by sequential chromatography on RET- and glutathione-S-transferase-coupled agarose columns.¹³ We also used antiphospho-Src homologous and collagen (Shc) (#Y317), which recognizes Shc proteins phosphorylated at Y317 (Upstate Biotechnology, Inc., Lake Placid, NY, USA); anti-phospho-MAPK (#9102), which is specific for p44/42MAPK [extracellular regulated kinases 1 and 2 (ERK1/2)] phosphorylated at Thr202/Tyr204; and antiphospho-MAPK/ERK kinase 1 and 2 (MEK1/2) (#9121), which is specific for MEK1/2 phosphorylated at Ser217/Ser221 (both from Cell Signalling, Beverly, MA, USA). Monoclonal antiphosphotyrosine (4G10) was purchased from Upstate Biotechnology, Inc. Secondary horseradish peroxidase-coupled antibodies were from Amersham Pharmacia Biotech (Little Chalfort, UK, USA). Immunoprecipitation and immunoblotting were performed according to standard procedures.¹⁴

Molecular constructs

Constructs used in this study encode the RET-9 isoform and were cloned in pBabe vectors. The wild-type RET (wtRET), C634R/RET and V804M/RET constructs are described elsewhere.¹⁵ V292M/RET (GTG>ATG) was generated by site-directed mutagenesis (QuickChange kit; Stratagene, La Jolla, CA, USA) and verified by DNA sequencing.

Cell transfection experiments

NIH3T3 fibroblasts were grown in DMEM containing 5% calf serum (GIBCO, Paisley, PA, USA). Stable NIH3T3 transfections were performed by calcium phosphate precipitation, as described elsewhere.¹⁵ The efficiency of marker-selected colony formation was roughly 50 colonies per pmol DNA. Transient transfections (luciferase assays) were carried out with the lipofectamine reagent according to the manufacturer's instructions (GIBCO). Parallel transfection with Green Fluorescent Protein (GFP) showed that about 20% of the cells expressed exogenous DNA.

Reporter assays

Firefly luciferase reporters were kindly provided by S. J. Gutkind (NIH, Bethesda, MD, USA). The pGL3-AP-1 (activator protein 1) and pGL3-SRF (serum response factor) reporters contain five repetitions of the target sequence (AP-1 and SRF, respectively) upstream to the firefly luciferase cDNA. Twenty-four hours after seeding, cells were transiently transfected in triplicate with one of

Table 1. Laboratory findings for the patient on admission

Parameter	Patient	Normal values
Urinary adrenalin (µg/24 h)	172	1.4–15.5
Urinary noradrenalin (µg/24 h)	623	12–85
Serum calcium (mmol/l)	2.37	2.17–2.67
Serum phosphate (mmol/l)	1.26	0.87–1.45
Calcitonin (pg/ml)	16.7	<20

Bold values indicate parameter out of the normal range.

the reporters plus the internal-control pRL-null plasmid expressing *Renilla* luciferase (Promega Corporation, Madison, WI, USA). Forty-eight hours post-transfection cells were serum-starved overnight. Luciferase activities were assayed with the Dual-Luciferase Reporter System (Promega Corporation). In all cases, the total amount of transfected plasmid DNA was normalized with empty vector DNA.

Results

The family history was negative for thyroid, parathyroid and adrenal disease, and direct sequencing of *RET* exons 10, 11, 13, 14, 15 and 16, and later, exons 8 and 18, revealed no germ-line or somatic mutations in the patient, although heterozygous single nucleotide polymorphisms were identified in exons 11 (Gly691Ser, GGT>AGT), 15 (Ser904Ser, TCC>TCG) and 18 (Arg982Cys, CGC>TGC) in both peripheral blood and tumour DNA. The pheochromocytoma was also negative for *SDHB*, *SDHD* and *VHL* mutations (data not shown).

Subsequent extension of *RET* analysis to exon 5 revealed a novel heterozygous germ-line mutation at codon 292 (Val292Met, GTG>ATG) (Fig. 1). The alteration was absent in 100 healthy controls (data not shown) and in the patient's mother and sister (the only relatives available for genetic testing).

The *in vitro* functional characteristics of the V292M/*RET* mutation are summarized in Fig. 2. Its transforming capacity was assessed in mass populations of marker-selected NIH3T3 cells

transfected with V292M/*RET*, wt-*RET* or C634R/*RET*, a strongly oncogenic mutant. As shown in Fig. 2b, V292M/*RET* transfectants correctly synthesized M_r 145 000 and M_r 160 000 *RET* isoforms, the former representing an immature precursor, the latter a mature glycosylated protein present on the cell surface.¹⁶ We also measured the *in vivo* tyrosine phosphorylation levels of the different *RET* proteins as read-outs of their oncogenic activation status. Unlike the wild-type protein, V292M/*RET* displayed detectable phosphotyrosine reactivity, but it was approximately 10 times weaker than that observed in C634R/*RET* (Fig. 2b). The new mutation's ability to activate intracellular signalling was reflected by higher than wild-type levels of phosphorylated MEK, MAPK and Shc adaptor [which is recruited by phosphorylated RET Y1062 and stimulates MEK and MAPK (ERK) kinase activity¹⁶ (Fig. 2c)]. Analysis of the proliferation rates of transfected and untransfected NIH3T3 cells under conditions of serum deprivation (1% calf serum) revealed mitogenic effects for both *RET* mutants, but those of C634R/*RET* were clearly stronger (Fig. 2d). In luciferase reporter assays (Fig. 2e), we examined also the activity of class A V804M/*RET* mutant: all the three mutants stimulated transcription factors known to be activated by *RET*. However, the activity of V292M resulted higher than wild-type *RET* but lower not only than the potent C634R but also than the less potent V804M mutant (Fig. 2e). Evidence of disulphide-bridge-mediated *RET* dimerization was observed only in C634R/*RET* mutants (data not shown).

Collectively, these findings indicate that the V292M mutation is a gain-of-function alteration with weaker oncogenic potential than

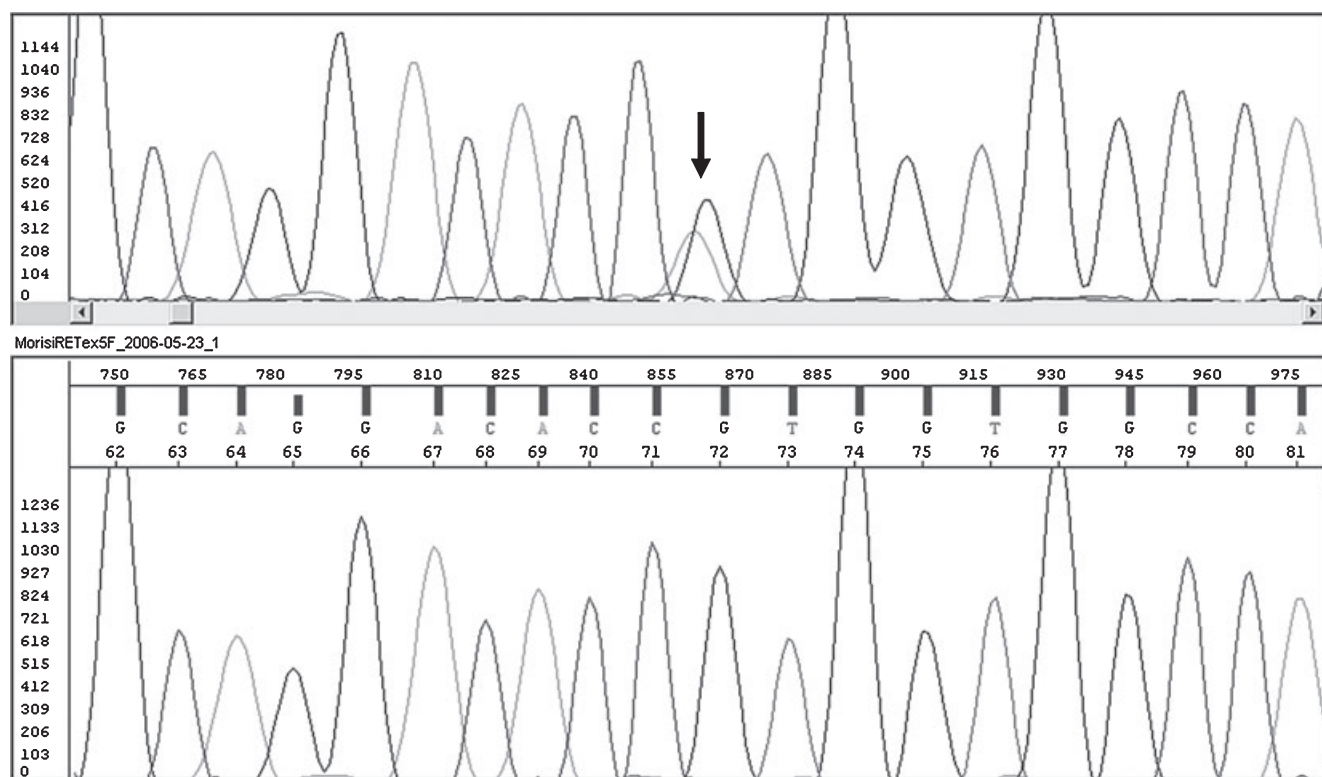


Fig. 1 Sequence electropherogram for mutation found in exon 5 of *RET*. The mutation (Val292Met, GTG>ATG) is marked by an arrow, and the wild-type sequence is shown below for comparison.

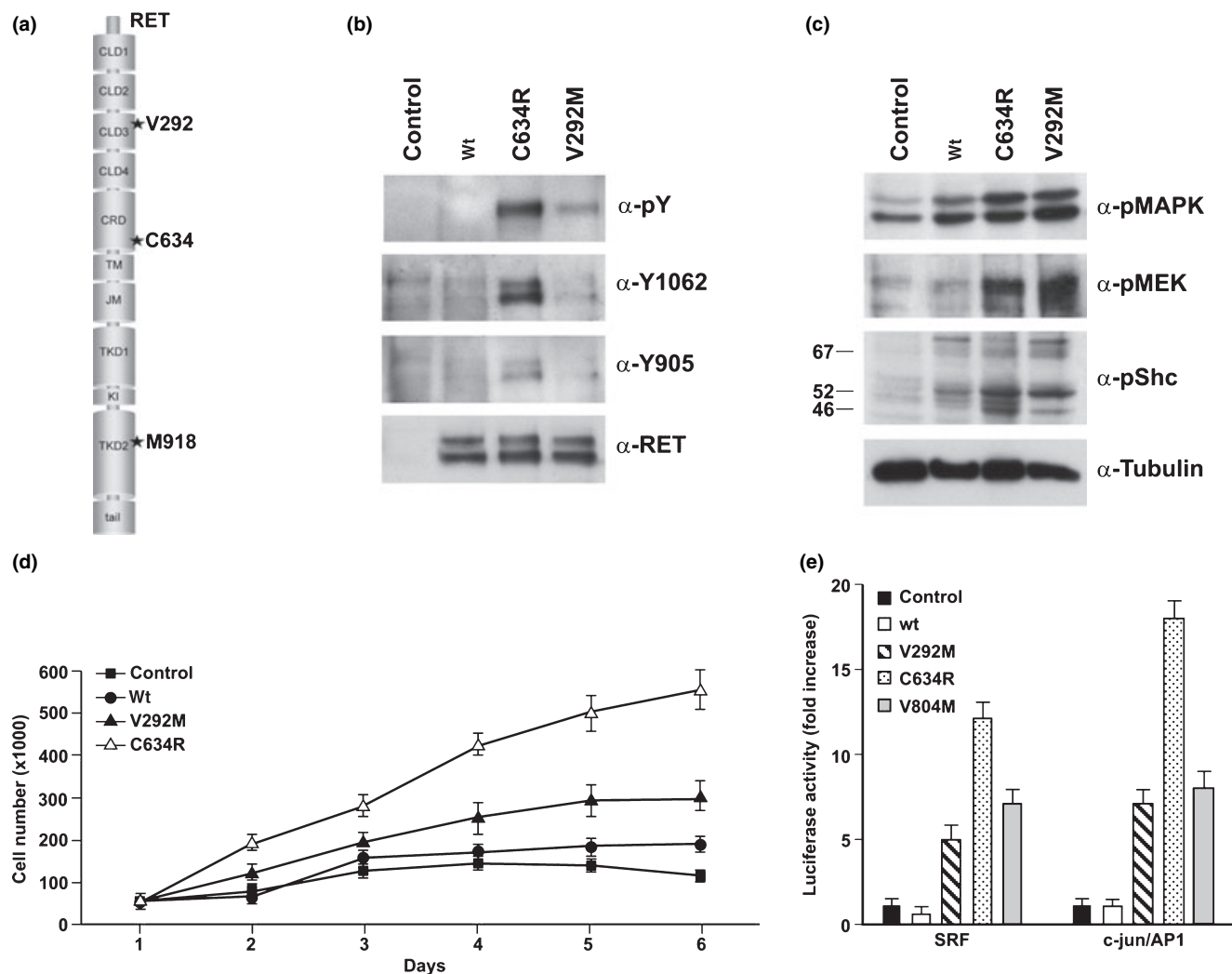


Fig. 2 Results of *in vitro* tests performed to characterize the V292M/RET mutation. (a) Schematic representation of the RET protein showing cadherin-like (CLD), cysteine-rich (CRD), transmembrane (TM), juxtamembrane (JM) and tyrosine kinase (TKD) domains and the locations of the V292M mutation and two other mutations commonly associated with medullary thyroid cancer. (b) Expression levels and phosphorylation status of wild-type (wt) and V292M and C634R mutant RET proteins in mass populations of stably transfected NIH-3T3 cells. Protein (500 µg) extracts from each cell line were immunoprecipitated with anti-RET and blotted with anti-RET, phosphorylation-specific (pY1062 and pY905) RET or phospho-tyrosine (pY) antibodies. (c) Protein (100 µg) extracts from the three transfected cell lines were blotted with phosphorylation-specific MAPK, MEK and Shc antibodies. Tubulin is shown for normalization. (d) Growth curves of NIH3T3 transfected with empty vector (control), wild-type (wt) RET and V292M and C634R RET mutants. Cells ($n = 50\,000$) were plated and counted at different time points. (e) Cells were transfected with the indicated plasmids plus V804M RET mutant together with the luciferase reporters driven by RET-activated transcription factors, SRF and AP1. Luciferase activity is expressed as fold increase of the activity seen in mock-transfected (control) cells. All data reported above are means (d, e) or representative (b, c) of the results obtained in three independent assays. Standard deviations never exceeded 20%.

C634R/RET or V804M/RET. Indeed, the number of transformed foci induced by C634R/RET in NIH3T3 cells was considerably higher than that observed in V292M/RET transfectants (data not shown).

Discussion

Genetic analysis now plays integral parts in the management of MTC and pheochromocytoma.^{5,17} The association of these two tumours is quite common in MEN-2A, but the fact that our patient's presenting symptoms were related to the adrenal rather

than the thyroid tumour is atypical, and the family history was negative for both types of disease. Furthermore, our initial sequencing studies disclosed no germ-line or somatic mutations involving RET regions commonly (exons 10, 11, 13, 15 and 16) or less commonly (exons 8 and 18) altered in MEN-2 syndromes. For these reasons, we also excluded the presence of mutations in genes – other than RET – associated with familial or sporadic pheochromocytoma (i.e. SDHB, SDHD and VHL).¹⁷

We finally discovered a heterozygous germ-line mutation in RET exon 5, which has never been described before. Pedigree analysis and the results of genetic testing in the patient's family (limited to

two of the four subjects at risk) indicate that the V292M/*RET* mutation is probably a *de novo* germ-line alteration carried only by the proband.

The location of the new mutation was surprising. The *RET* mutations associated with MEN-2 thus far almost invariably target the extracellular cysteine-rich box adjacent to the plasma membrane (encoded by exons 10 and 11) or the intracellular tyrosine kinase domain (Fig. 2a). Our patient's exon 5 mutation involves one of the receptor's four extracellular cadherin-like repeats. There is only one other report linking MEN-2 to a *RET* mutation involving this region:¹⁸ it involved a missense glycine for arginine substitution at codon 321 (also in exon 5) found in four members of a three-generation Czech kindred with FMTC (one with MTC, one with C-cell hyperplasia and two with no evidence of either). The authors' assumption that this mutation causes FMTC was based exclusively on these findings and the documented absence of mutations in the hot-spot *RET* exons.¹⁸

Legitimate questions can be raised regarding the significance of this mutation and the one found in our patient. Indeed, on a theoretical basis, the hypothesis of a very rare polymorphism cannot be definitely ruled out, even though the V292M/*RET* mutation did not occur in any of the 100 healthy controls. To address this issue, we investigated the biological activity of V292M/*RET* mutant constructs *in vitro*. The results are clearly indicative of a gain-of-function mutation although the molecular mechanism underlying its activating effects remains unknown. The classic extracellular cysteine-targeting *RET* mutants promote aberrant inter-chain disulphide bonding, which mimics ligand-induced *RET* homodimerization.¹⁵ By contrast, the constitutive *RET* activation produced by the V292M mutant is dimerization-independent, which is not surprising as it does not affect any of the cysteine residues. Unfortunately, the structure of the *RET* extracellular domain has not been solved yet, and therefore it is difficult to figure out what type of folding effect may the V292M mutation have. However, it is worth noting that many other examples of gain-of-function mutations targeting noncysteine residues of the extracellular domain of receptor tyrosine kinases other than *RET* have been reported in tumours.^{19,20} The extracellular cadherin-like domains are thought to play roles in *RET*'s interaction with its ligands,²¹ but our findings demonstrate that alterations in this region can also lead to ligand-independent *RET* activation.

RET point mutants associated with MEN-2A (or MEN2-B) generally display strong transforming potential; those found in FMTC

patients are consistently less potent.^{4,16} Our *in vitro* analysis indicates that V292M/*RET* is a substantially weaker transforming mutant than C634R/*RET* or V804M/*RET*. This finding suggests that other genetic determinants may also have contributed to the tumour formation in our patient. Little is known about the impact of genetic modifiers on MEN-2 phenotypes, but the syndrome's variable clinical presentation has been putatively linked to factors capable of modifying the transformation process.²² More recently, wide variability in the activated *RET*-driven MTC phenotype has been reported in transgenic mice with different genetic backgrounds: penetrance rates in the different strains ranged from 0% to 98%, and there were also significant differences in tumour size.²³

Susceptibility to MTC seems to be influenced by certain *RET* polymorphisms.^{24–28} Their role in the development of the patient's tumours is, however, difficult to prove. Polymorphisms involving genes whose products interact with *RET* or components of the *RET* signalling pathway have also been reported to modify the risk of MTC.^{12,27} Phenotype-modifying effects might also stem from secondary genetic alterations at the somatic level, which act in concert with the germ-line *RET* mutations to trigger MTC development.^{1,29} The second hit can involve loss of the wild-type *RET* allele or duplication of the mutated one³⁰ – events that can be excluded in the present case – or deletion and amplification of entire chromosomes.³¹

In conclusion, this case illustrates the importance of the American Thyroid Association's recent recommendation to extend *RET* sequencing studies to the gene's entire coding region when initial testing of a patient with clinical signs of MEN-2 fails to reveal *RET* hot-spot mutations or when genotype-phenotype discrepancies emerge.⁵ Re-testing may be warranted in certain cases with MEN-2 phenotypes currently classified as *RET*-mutation-negative. Failure to identify the causative mutation has an enormous impact on the management of these patients and their families, in terms of prognosis, quality of life and health-care costs. Functional characterization of newly identified *RET* mutants is important for estimating their contribution to cancer-susceptibility phenotypes. The mechanism by which V292M activates *RET* remains to be defined, and the current data suggest that it may require the contributions of other genetic or epigenetic determinants to produce predisposition to cancer.

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Conflicts of interest

Authors have nothing to declare.

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Table 2. Primers used for *RET* sequencing

Gene	Exon	Forward primer	Reverse primer	Temp. (°C)
<i>RET</i>	5	gacgtgcagcattctaaggt	catgaagagcgagcacctca	60
	8	gctgttccctgtccttgg	ctatgtggcatcgagagc	58
	10	tatgcttgcgacaccagttg	gatgtgctgttgagacctct	64
	11	ccatgaggcagagcatagc	cctcctctgccagcgttg	60
	13	ctctctgtctgaacttgggc	agaacaggcgctgtatggagc	61
	14	tggctcctggaagacca	gtgggctagagtgtggca	58
	15	ctctgtgtgtcacaccaggc	gcactgcctgcccatggt	64
	16	agggatagggcctggccttc	taacctccacccaagagag	62
	18	gctgtccttctgagacctg	cacactgggaactctgagg	58

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